J. Biochem. 120, 1153-1162 (1996)

Glycation Decreases the Stability of the Triple-Helical Strands of Fibrous Collagen against Proteolytic Degradation by Pepsin in a Specific Temperature Range

Sheng-Fang Tian,* Shuji Toda,† Hideaki Higashino,‡ and Sueo Matsumura*,[1]

*Departments of *Biochemistry and †Pathology, Saga Medical School, Saga 849; and ‡Department of Pharmacology, Kinki University School of Medicine, Osaka-Sayama 589*

Received for publication, July 18, 1996

When fibrous collagen of rat tail tendons was glycated by incubation with ribose, it became highly insoluble in dilute acetic acid and resistant to pepsin digestion at 5°C, since it was cross-linked by advanced glycation end products. Extensively glycated fibrous collagen was found to be much less stable than non-glycated control fibrous collagen against pepsin digestion at 30°C. Under conditions where nearly all of the glycated fibrous collagen was degraded into small peptides by pepsin, approximately 45% of the control collagen was left as large polypeptides having nearly the whole length of its triple-helical region. A soluble collagen, which consisted primarily of the triple-helical region of monomeric collagen, was found to be glycated as efficiently as the fibrous collagen on incubation with ribose at 30°C, while the rate of cross-linking of the soluble collagen was very low, suggesting that the triple-helical strands do not undergo intramolecular cross-linking and that most of the cross-links produced in the glycated fibrous collagen are intermolecular ones. The glycated soluble collagen was as stable as the control collagen against pepsin digestion at 30°C. These results indicate that the triple-helical strands of glycated fibrous collagen are much less stable than those of the non-glycated form against proteolytic digestion by pepsin at a temperature close to but below their melting point. Sugar-derived intermolecular cross-links are supposed to underly the decreased stability of the triple-helical strands.

Key words: advanced glycation end products, collagen, cross-link, glycation, pepsin digestion.

Collagen normally undergoes a series of age-related changes characterized by decreasing solubility, increasing stiffness and increasing resistance to enzymatic digestion (*1*), and these changes are known to be accelerated in diabetes mellitus (*1-6*). These age-related and diabetes-accelerated changes of collagen are supposed to be brought about by sugar-derived cross-links recognized also as advanced glycation/glycosylation end products (AGE), which appear to be produced through a series of non-enzymatic reactions between the side chains of collagen polypeptides and glucose or its metabolites (*3-10*). Pentosidine, a fluorescent imidazopyridinium compound including a lysine and an arginine cross-linked by a derivative of "pentose," has been proposed to be the major senescence cross-link responsible for the age-related and diabetes-related changes in the physical properties of collagen (*7-10*), while a recent study suggested that a non-fluorescent cross-link other than pentosidine is responsible for the changes (*11*). Ribose, which gives a much larger proportion of the aldehyde form than glucose in solution (*12*), has been shown to be far more effective in glycating collagen *in vitro* (*11, 13-15*), as well as in forming pentosidine (*7-9, 11*) and the non-fluorescent cross-link *in vitro* (*11*).

Fibrous collagen glycated *in vitro* by incubation with glucose or ribose has been shown to exhibit increased tensile stress and stiffness, increased thermal stability in a urea solution, and increased resistance to pepsin digestion compared to non-glycated fibrous collagen (*6, 13, 15-17*). An electron microscopic study has shown that collagen fibrils in glycated collagen fibers exhibit larger and irregular diameters, and closer packing compared to those in control collagen fibers (*18*). An X-ray diffraction study has also shown that glycation induces expansion of the intermolecular spaces of collagen fibrils (*14*). Whereas these studies have demonstrated a few glycation-induced changes in the organization of the fibers and fibrils of collagen, it is not known how glycation affects the conformation and stability of individual collagen molecules.

The type I collagen molecule is composed of elongated triple-helical polypeptide chains, *i.e.* two $\alpha 1$ and one $\alpha 2$ chains, with small non-helical regions at both their N- and C-termini (*19, 20*). The enzymatic cross-links, hydroxylysylpyridinium and lysyl pyridinium, which are produced through the action of lysyl oxidase during the maturation of collagen fibrils, are localized primarily in the non-helical regions (*21, 22*), while the non-enzymatic cross-links of AGE are supposed to be localized primarily in the triple-

[1] To whom correspondence should be addressed at: Department of Basic Medical Sciences, Faculty of Health Science, Kobe University School of Medicine, Suma, Kobe 654-01. Tel: +81-78-796-4590, Fax: +81-78-796-4589

Abbreviations: AGE, advanced glycation/glycosylation end products; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid.

helical regions (*14, 15*). The triple-helical regions of collagen are resistant to pepsin digestion at a low temperature where the triple-helical strands do not unfold, while the non-helical regions of collagen are labile against pepsin digestion even at a low temperature (*19, 23*). The present study was undertaken to determine how glycation affects the molecular conformation of the triple-helical strands of collagen using pepsin as a probe. In contrast to the previous understanding, we have found that glycation decreases the stability of the triple-helical strands of collagen against proteolytic degradation by pepsin in a specific temperature range rather than protects them from degradation.

MATERIALS AND METHODS

Materials—Tail tendons dissected from 6-month-old Wistar rats were washed with phosphate-buffered saline (10 mM sodium phosphate, pH 7.5, containing 0.15 M NaCl and 8 mM NaN_3) and distilled water, and then lyophilized. The tendons were cut into about 2-mm pieces and used for the present studies. D-[1-^{14}C]Ribose (55 mCi/mmol) was obtained from American Radiolabeled Chemicals. Soluble collagen, consisting primarily of monomeric collagen, was prepared as follows. Pieces of rat tail tendon were digested with 1/20 weight of pepsin in 0.5 M acetic acid for 5 days at 25°C (*15*). The pepsin digest was subjected to salt fractionation in acidic and neutral solutions as described by Miller and Rhodes (*23*). The monomeric collagen sample was dialyzed against the phosphate-buffered saline and then centrifuged for 60 min at 20,000 × *g*. The supernatant was used as "soluble collagen." The α-chains of collagen were isolated from the soluble collagen by Sepharose CL-6B gel filtration in the presence of 2 M guanidine-HCl as described by Miller and Rhodes (*23*). The α-chain fractions were pooled, dialyzed several times against a large excess volume of distilled water by replacement with fresh distilled water, and then lyophilized. These α-chains were used as reference collagen chains for determination of the concentrations of collagen chains in various samples.

Glycation of Fibrous Collagen of Rat Tail Tendons—Pieces of tendons were suspended at a concentration of 5 mg/ml in the phosphate-buffered saline containing 0.2 M D-ribose and then incubated at 37°C for the indicated periods of time, unless otherwise noted (*15*). The tendon pieces were washed several times with the phosphate-buffered saline and then subjected to the variety of analytical procedures described below. Where indicated, 2 mg of the tendon pieces was incubated with 0.4 ml of the phosphate-buffered saline containing 0.2 M [^{14}C]ribose (12.5 μCi/mmol) for the indicated period (*15*), and followed by washing 4 times with 5 ml of the phosphate-buffered saline. These tendon pieces were further washed 3 times with 5 ml of ethanol on a glass fiber filter (Whatman GF/F), dried under a tungsten lamp, and then immersed in 0.5% 2,5-diphenyloxazole in toluene. The radioactivity was determined with a scintillation counter. The molecular weights of the α1 and α2 chains of collagen were assumed both to be 100,000.

Enzymatic and Non-Enzymatic Solubilization of Fibrous Collagen—Ten-milligram pieces of control or glycated tendons were immersed in either (i) 2 ml of 0.1 N NaOH, (ii) 5 ml of 0.5 M acetic acid, (iii) 2 ml of 2% SDS, 5% 2-mercaptoethanol, and 0.1 M Tris-HCl, pH 6.8, or (iv) 2 ml of 0.5 M acetic acid containing 0.5 mg of pepsin (Sigma). The samples in 0.1 N NaOH were boiled for 30 min and then neutralized with 6 N HCl. The samples in 0.5 M acetic acid were incubated for 2 days at 25°C and then centrifuged for 60 min at 20,000 × *g*. The samples in the SDS solution were incubated for 10 h at 45°C, boiled for 30 min, and then centrifuged as above. The samples in 0.5 M acetic acid containing pepsin were incubated for the indicated periods of time at the indicated temperatures and then centrifuged as above. The precipitates were washed several times with distilled water and then lyophilized. The supernatants and precipitates were subjected to the various analytical procedures described below.

Analytical Procedures—The concentration of collagen was determined by measuring the content of hydroxyproline as described by Berg (*24, 25*), and the content of hydroxyproline in type I collagen was assumed to be 14% (w/w) (*26*).

AGE were determined by measuring the absorbance of the NaOH hydrolysates of collagen at 400 nm.

SDS-PAGE was performed as described previously (*25, 27*). SDS-PAGE performed with gels comprising only a separation gel gave nearly identical electrophoretic patterns to that obtained with standard gels consisting of both separation and stacking gels (*27*). To quantify the monomeric and oligomeric chains of collagen, the Coomassie Blue-stained gels were dried, and then the α-chain and β-chain regions of collagen were cut out from the gels and immersed in 1.5 ml of 25% pyridine (*25, 28*). The absorbance of the Coomassie Blue eluted from the gels was determined by reading at 605 nm.

To determine the molecular mass of collagen in pepsin digests, the digests were adjusted to pH 7.5 with 6 N NaOH, boiled for 30 min with 2% SDS, and then subjected to gel filtration on a Sepharose CL-6B column equilibrated with 20 mM Tris-HCl, pH 7.5, 0.1 M NaCl, and 0.1% SDS.

Protease activity of pepsin was determined using bovine serum albumin as a substrate as described below. Pepsin was dissolved to a concentration of 0.25 mg/ml in 0.5 M acetic acid or 0.5 M acetic acid containing 2 mg/ml of rat tail tendon collagen, and then incubated at 30°C. At the times indicated, 5 μl of each incubation mixture was transferred to a 2-ml incubation mixture containing 0.5 M acetic acid and 1 mg/ml of bovine serum albumin, and then incubated at 25°C. At 10 min, 0.5 ml of 60% (w/v) perchloric acid was added to the mixtures, and then the mixtures were centrifuged for 10 min at 10,000 × *g*. The absorbance of the supernatants was measured at 280 nm.

Glycation of Soluble Collagen—Soluble collagen was glycated with 0.2 M ribose or [^{14}C]ribose under the same conditions as described above for the tendon pieces except that the concentration of collagen was 2.0 mg/ml and the incubation temperature was 30°C, since the soluble collagen formed gels of collagen fibrils when incubated at a higher concentration or at a higher temperature (*29*). The soluble collagen glycated with non-radioactive ribose was dialyzed against the phosphate-buffered saline and then subjected to the analytical procedures described below. The collagen glycated with radioactive ribose was made insoluble by the addition of 20% TCA, collected on a glass fiber filter, and washed 5 times with 5 ml of 20% TCA and then twice with 5 ml of ethanol. The radioactivity was determined as described above.

RESULTS

Effects of Glycation on the Solubility of Fibrous Collagen in Dilute Acetic Acid and SDS Solutions—Rat tail tendons, comprising nearly homogeneous fibers of type I collagen, were incubated with 0.2 M [^{14}C]ribose at 37°C for various periods, ranging from 0 to 30 days, and the time course of glycation of collagen was determined (Fig. 1A). Approximately 6 mol of ribose was incorporated into 1 mol of α-chains of collagen in 2 days, and the glycation continued to increase gradually up to about 20 days, when approximately 16 mol of ribose was incorporated into 1 mol of α-chains. Since glycation was reported to occur initially at the lysyl residues of proteins (*7-9, 22*), and type I collagen of rat contains about 28 mol of lysine per mol α-chains (*19*), approximately 60% of the lysyl residues of the tendon collagen were supposed to be glycated under the conditions examined. The content in the fibers of brown-colored materials, which appeared to be derived from AGE, also appeared to increase with increasing incubation time, in parallel with the progress of glycation (Fig. 1A). With increasing incubation time, the collagen fibers became highly insoluble in dilute acetic acid; whereas the control collagen fibers were solubilized almost completely in dilute acetic acid, and the collagen fibers that had been incubated with ribose for 2 days or longer were almost completely insoluble in dilute acetic acid (Fig. 1B). Since dilute acetic acid is known to dissolve collagen fibers by disrupting the non-covalent interactions among both the fibrils and the molecules, but not those among the triple-helical chains (*19, 23*), the result appeared to suggest that sufficient numbers of intermolecular and/or interfibrillar cross-links were introduced into the fibers on incubation with ribose for 2 days to prevent their dissociation in acetic acid. When these glycated fibers were boiled with an SDS solution to disrupt the non-covalent interactions among the triple-helical chains as well as the non-covalent interactions among the fibrils and molecules, the tendons glycated for less than 4 days were solubilized efficiently, while those glycated for longer periods were solubilized partially, and those glycated for longer than 20 days were almost completely resistant to solubilization (Fig. 1B). When the SDS-boiled extracts were subjected to SDS-PAGE, the extract of the control tendons was found to contain high concentrations of monomeric (α) and dimeric (β) chains of collagen, which were estimated to account for about 32 and 48% of total collagen in the tendons, respectively (Fig. 2). On the other hand, the extracts of the glycated tendons contained much lower concentrations of these chains; the concentration of α-chains in the extract of the tendons glycated for 2 days was approximately 1/3 of that in the control extract, and the extracts of the tendons glycated for longer than 6 days did not contain appreciable concentrations of α-chains (Figs. 1B and 2). These results indicated that nearly all of the collagen chains were cross-linked initially to polymeric

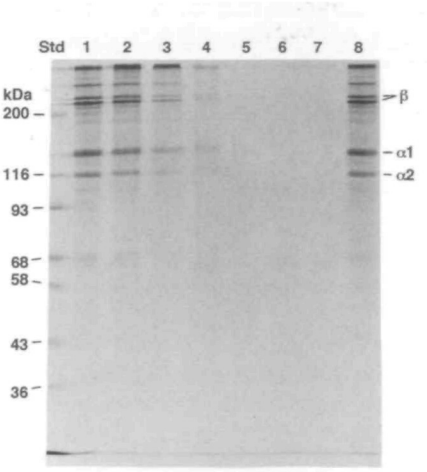

Fig. 2. **SDS-PAGE of SDS-boiled extracts of control and glycated rat tail tendons.** Rat tail tendons were incubated at 37°C with 0.2 M ribose for the indicated numbers of days and then extracted with an SDS solution as in Fig. 1B. The extracts were subjected to SDS-PAGE. The numbers of days of incubation with ribose were: lane 1, 0 days; lane 2, 1 day; lane 3, 2 days; lane 4, 4 days; lane 5, 6 days; lane 6, 8 days; lane 7, 14 days; and lane 8, 0 days. Std, molecular mass standards in kDa. α1, α1 chains of collagen; α2, α2 chains of collagen; β, β-chains of collagen.

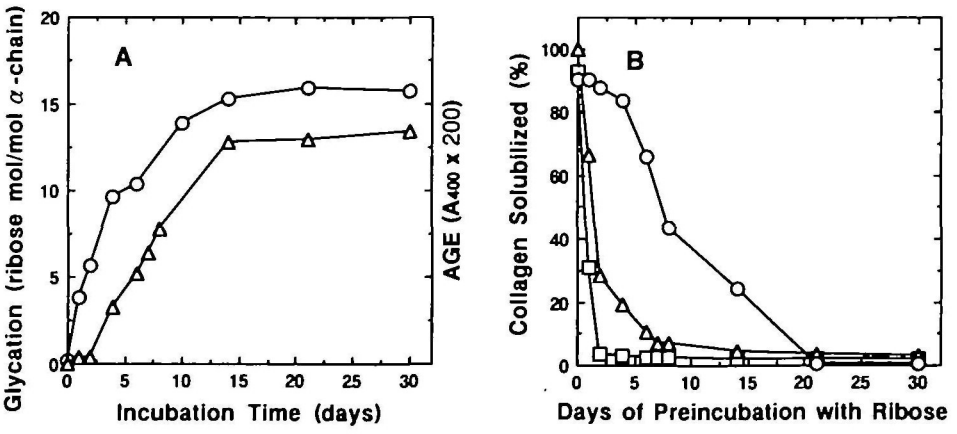

Fig. 1. **Glycation of fibrous collagen of rat tail tendons, and glycation-induced changes in the solubility of collagen and contents of the monomeric chains.** (A) Rat tail tendons were incubated with 0.2 M [^{14}C]ribose at 37°C for the indicated numbers of days, and the contents of radioactive ribose covalently bound to the collagen were determined as described under "MATERIALS AND METHODS" (○). Rat tail tendons that had been incubated with 0.2 M non-radioactive ribose for the indicated priods were solubilized by boiling with 0.1 N NaOH, and then the absorbance of the colored materials (AGE) was measured at 400 nm (△). (B) Rat tail tendons that had been incubated with 0.2 M ribose as above were suspended in a 0.5 M acetic acid (□) or 2% SDS (○) solution. The samples in 0.5 M acetic acid were incubated for 2 day at 25°C and then centrifuged for 60 min at 20,000 × *g*. The samples in the SDS solution were incubated for 10 h at 45°C, boiled for 30 min, and then centrifuged as above. The supernatants were assayed for collagen by measuring their hydroxyproline contents. The SDS-boiled extracts were subjected to SDS-PAGE (see Fig. 2), and the Coomassie Blue-stained α-chains were determined as described under "MATERIALS AND METHODS" (△).

polypeptides, that were unable to enter the gel, and then to highly polymeric polypeptides, that were insoluble in a hot SDS solution, on incubation with ribose for 6 days and longer, respectively.

Effects of Glycation on the Digestability of Fibrous Collagen by Pepsin—Although it is well accepted that glycated collagen fibers are resistant to pepsin digestion at a low temperature such as 5°C and it has also been reported that glycated collagen fibers are degraded partially at 37°C by pepsin (*17*), it is not known at which temperature the fibers become digestable by pepsin. In order to determine the temperature at which glycated collagen fibers become digestable by pepsin, collagen fibers glycated for 10 days were incubated with pepsin at various temperatures, and the time courses of degradation of the fibers were determined by measuring the concentration of soluble collagen released from the fibers (Fig. 3). When the glycated fibers were incubated with pepsin at 5°C, they were fairly resistant to pepsin digestion, less than 10% of the collagen being degraded into soluble (poly)peptides even after 30 days. These fibers were found to become partially digestable by pepsin at 25°C. It was further found that the fibers became effectively digestable at 30°C, about 80% of the fibers being degraded into soluble peptides in 5 days. When collagen fibers glycated for 20 days were incubated with pepsin at 30°C, the fibers were also found to be degraded nearly completely in about 20 days. To clarify the relationship between the duration of glycation of the collagen fibers and the extent of their degradation by pepsin at 5, 25, and 30°C, rat tail tendons glycated for various periods were incubated for 30 days with pepsin at the respective temperatures, and then the extents of degradation of the fibers were determined (Fig. 4). The collagen fibers glycated for longer than 6 days were highly resistant to pepsin digestion at 5°C, less than 10% of the collagen being degraded into soluble peptides. These fibers were also fairly stable against pepsin digestion at 25°C, less than 30 and 20% of the collagen being released from the fibers glycated for 6 and 21 days, respectively. On the other hand, when the glycated fibers were incubated with pepsin at 30°C, all of the fibers, including those glycated for 21 days, were degraded almost completely. These results indicate that glycated collagen fibers are digestable by pepsin even at 30°C, a temperature at which the triple-helical chains of collagen do not appear to unfold to a significant extent (*30*).

Effect of Prolonged Incubation on the Proteolytic Activity of Pepsin—The results shown in Figs. 3 and 4 were obtained by incubating pepsin and collagen at and below 30°C for fairly long periods such as 30 days. To determine whether pepsin underwent a change in proteolytic activity during the incubation or not, pepsin was preincubated for various periods at 30°C in the presence or absence of collagen, and then the time course of the change in its proteolytic activity was determined using bovine serum albumin as a substrate. As shown in Fig. 5, pepsin that had been incubated in the absence of collagen was fairly stable for a long period, and pepsin preincubated for 30 days possessed approximately 80% of the protease activity of non-preincubated control pepsin. On the other hand, pepsin

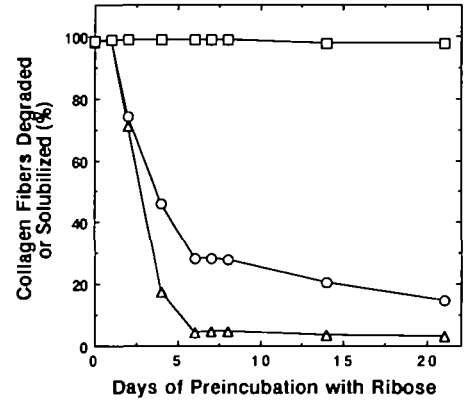

Fig. 4. **Effect of glycation time on the digestability of rat tail tendon collagen by pepsin at different temperatures.** Rat tail tendons that had been glycated for the indicated numbers of days with 0.2 M ribose were incubated with 1/20 weight of pepsin for 30 days at 5 (△), 25 (○), or 30°C (□), and then centrifuged for 60 min at 20,000×*g*. The supernatants were assayed for collagen.

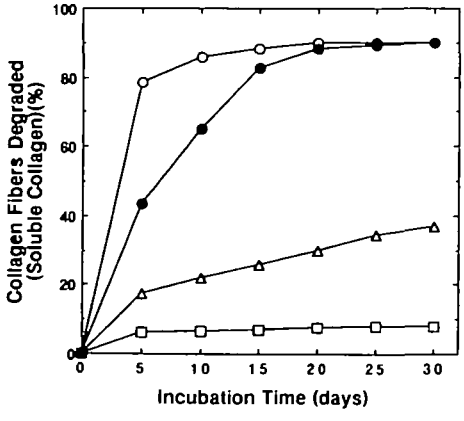

Fig. 3. **Time courses of degradation of glycated rat tail tendon collagen by pepsin.** Rat tail tendons that had been glycated with 0.2 M ribose for 10 (○, △, □) or 20 days (●) were incubated in 0.5 M acetic acid containing 1/20 weight of pepsin for the indicated numbers of days at 5 (□), 25 (△), or 30°C (○, ●), and then centrifuged for 60 min at 20,000×*g*. The collagen recovered in the supernatants was determined as an index of the degradation of the collagen fibers.

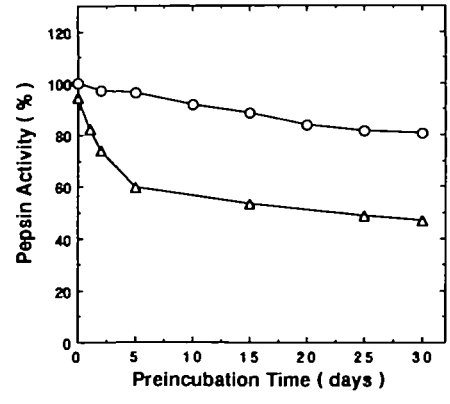

Fig. 5. **Effects of preincubation with and without collagen on the protease activity of pepsin.** Pepsin was dissolved to 0.25 mg/ml in 0.5 M acetic acid (○) or 0.5 M acetic acid containing 2 mg/ml of collagen (△), and then incubated at 30°C. Aliquots (5 μl) of the incubation mixtures were removed at the indicated time and assayed for the protease activity using serum albumin as a substrate, as described under "MATERIALS AND METHODS."

was found to lose approximately 40% of its protease activity on 5 days preincubation with collagen, while the activity was fairly stable thereafter. Pepsin that had been preincubated for 30 days with collagen had approximately 45% of the activity of control pepsin. It was apparent, therefore, that pepsin retained a sufficient level of proteolytic activity to further cleave the polypeptide chains of collagen even after 30 days incubation, if its cleavage sites were available. The rather slow rate of degradation of glycated fibrous collagen by pepsin at 30°C thus appeared to be related to its particular molecular conformation rather than the loss of its proteolytic activity. Although the reason why pepsin loses about 40% of its proteolytic activity shortly after incubation with collagen is not known, it is likely to be due to inhibition by the partial degradation products of collagen (see following experiments).

Characterization of the Degradation Products at 30°C of Control and Glycated Fibrous Collagens with Pepsin—To determine the molecular sizes of the degradation products with pepsin at 30°C of control and glycated fibrous collagen, tendons that had been glycated for various periods were incubated with pepsin for 30 days at 30°C, and then the digests were subjected to SDS-PAGE. As shown in Fig. 6, the pepsin digest of the non-glycated control fibers was found to contain a high concentration of monomeric collagen chains having a similar electrophoretic mobility to that of the α1 or α2 chains present in the control fibers prior to pepsin digestion (see Fig. 2). When the concentration of the α-chains in the control digest was measured as described under "MATERIALS AND METHODS," they were found to account for about 45% of the total collagen present in the control fibers, indicating that the triple-helical regions of non-glycated control collagen were fairly stable against prolonged pepsin digestion at 30°C. The pepsin digests of the tendons glycated for less than 8 days also contained appreciable concentrations of monomeric chains of collagen that migrated to slightly above the non-glycated α1 or α2 chains, while the digests of the tendons glycated for longer periods did not contain appreciable concentrations of these chains. The concentration of the small peptides at the front of the gel appeared to increase with increasing time of glycation. The pepsin digests of tendons glycated for 4 to 8 days contained considerable amounts of polymeric collagen, that migrated to the top of the gel, while such digests of tendons glycated for longer periods contained only low concentrations of collagen at the same position: This appeared to reflect one of the following two facts: (i) the long-glycated collagen was degraded by pepsin into small peptides more effectively than the short-glycated collagen. (ii) A large fraction of the degradation products of long-glycated collagen did not enter the gel because they still had too large molecular masses to enter the gel, although they had already been made soluble in dilute acetic acid and in an SDS solution. To determine which of the interpretations is the case, the contents of TCA-insoluble collagen or large polypeptides in the pepsin digests were determined for tendons glycated for various periods by adding 20% of TCA to the respective digests (Fig. 7). Whereas the pepsin digest of the control tendons contained more than 70% of TCA-insoluble, large polypeptides of collagen, those of the glycated fibers contained much smaller proportions of TCA-insoluble collagen, and the digest of the fibers glycated for 30 days contained less than 20% of TCA-insoluble collagen. It was apparent, therefore, that the pepsin digests of long-glycated collagen contained smaller proportions of large polypeptides than the non-glycated or short-glycated collagen. When the pepsin digests of the control and 20-day-glycated tendons were boiled in an SDS solution and then subjected to gel filtration on a Sepharose CL-6B column equilibrated with a buffer containing SDS, the digest of control tendons gave two peaks of UV-absorbing materials at fractions corresponding to about 100 kDa and smaller than 20 kDa, respectively, while the digest of the glycated fibers gave only one peak at the low-molecular mass fractions (Fig. 8). SDS-PAGE of the column fractions revealed that the first peak fractions of the control fibers

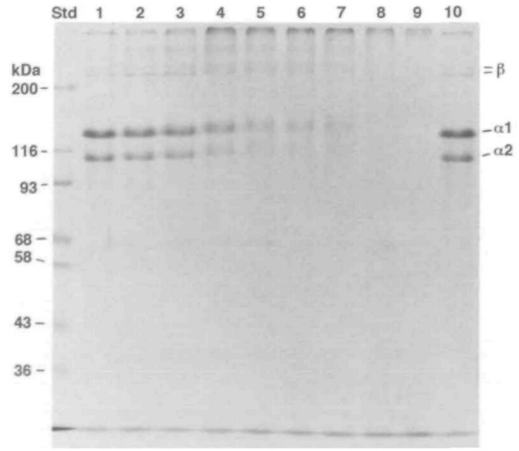

Fig. 6. **SDS-PAGE of pepsin digests of rat tail tendons glycated for various periods.** Rat tail tendons that had been preincubated with 0.2 M ribose for the indicated numbers of days at 37°C were incubated with pepsin at 30°C for 30 days, as in Fig. 4. The digests were subjected to SDS-PAGE. The periods of incubation with ribose were: lane 1, 0 days; lane 2, 1 day; lane 3, 2 days; lane 4, 4 days; lane 5, 6 days; lane 6, 7 days; lane 7, 8 days; lane 8, 14 days; lane 9, 21 days; and lane 10, 0 days. Std, molecular mass standards in kDa. α1, α1 chains of collagen; α2, α2 chains of collagen; β, β-chains of collagen.

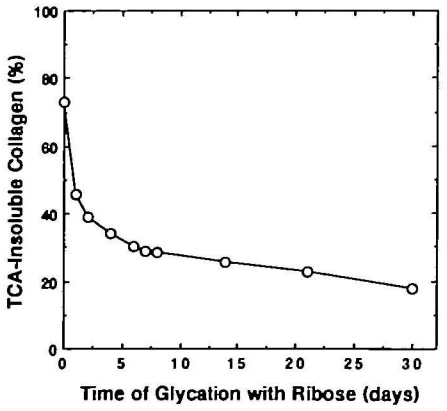

Fig. 7. **Effect of the glycation time on the degradation of rat tail tendon collagen by pepsin.** Rat tail tendons were preincubated with 0.2 M ribose for the indicated numbers of days at 37°C and then incubated with pepsin for 30 days at 30°C, as in Fig. 4. The pepsin digests were made to 20% with respect to TCA and then centrifuged for 10 min at 20,000×g to sediment the insoluble polypeptides. The contents of collagen in the TCA-insoluble pellets were determined.

contained $\alpha 1$ and $\alpha 2$ chains of collagen, while the second peak fractions from both control and glycated fibers contained only small peptides at the front of the gel (Fig. 9). These results indicated that nearly all of the long-glycated collagen, including its triple-helical regions, was degraded into small peptides on incubation with pepsin at 30°C for 30 days, while the triple-helical regions of control collagen were degraded only partially under the same conditions.

Pepsin Digestion of Control and Glycated Fibrous Collagens at 35°C—Although the results described above revealed that the triple-helical regions of control collagen were fairly stable against pepsin digestion at and below 30°C, these regions were found to become very labile when the temperature was raised to 35°C, the melting point of the triple-helical strands in dilute acetic acid (*30*). When the control collagen, that had been preincubated with pepsin for 30 days at 30°C, was reincubated at 35°C, the triple-helical chains were degraded into small peptides within 2 to 3 h (Fig. 10). When control fibrous collagen was directly subjected to pepsin digestion at 35°C, most of it was degraded into TCA-soluble small peptides within about 20 h. On the other hand, when the 20-day-glycated fibrous collagen was subjected to pepsin digestion at 35°C, it took about 60 h to degrade most of the glycated fibrous collagen into small peptides. These results indicate that the triple-helical strands of both control and glycated collagens become very labile against pepsin digestion at the melting point of the triple-helical strands, and that the rate of degradation of the control collagen is higher than that of the glycated collagen at this temperature, as reported previously (*17*). Highly cross-linked collagen thus appeared to be much less accessible to pepsin than non-cross-linked collagen at and above its melting point.

Glycation of Soluble and Insoluble Collagens with Ribose—Although the results in Figs. 1B and 2 suggested that the fibrous collagen underwent "intermolecular" cross-linking on incubation with ribose, it was not known whether its triple-helical regions underwent "intramolecular" cross-linking or not. In order to determine whether the triple-helical regions undergo intramolecular cross-linking or not, a soluble collagen, which consisted almost only of the triple-helical regions of monomeric collagen and lacked most, if not all, of the intermolecular interactions at and

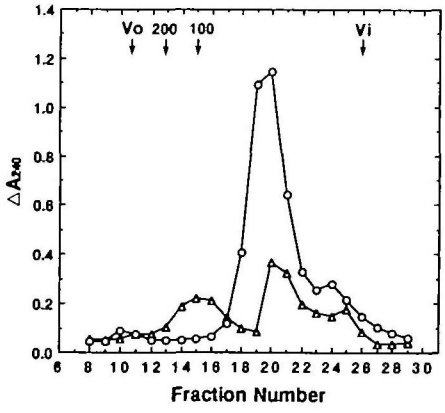

Fig. 8. **Sepharose CL-6B gel filtration of pepsin digests of control and glycated rat tail tendon collagen.** Control and glycat-ed rat tail tendons were incubated with pepsin for 30 days at 30°C. The pepsin digests were neutralized, boiled in 2% SDS, and then subjected to Sepharose CL-6B gel filtration as described under "MATERIALS AND METHODS." The absorbance of the column fractions was measured at 240 nm. Pepsin digest of control tendons (△); pepsin digest of tendons glycated for 20 days (○). V_o, void volume; V_t, included volume. The peak fractions in which SDS-boiled reference proteins (myosin heavy chain, 200 kDa; α-actinin, 100 kDa) were eluted are indicated by their molecular masses in kDa.

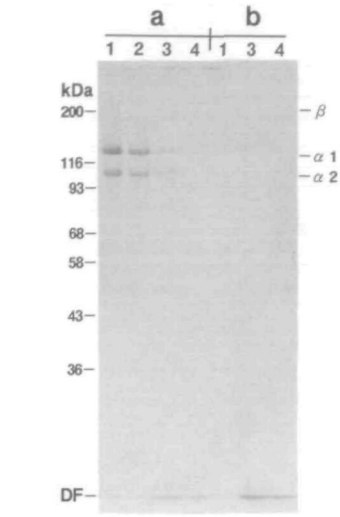

Fig. 9. **SDS-PAGE of Sepharose CL-6B column fractions of pepsin digests of control and glycated tendon collagens.** The column fractions of the pepsin digests of control (a) and glycated tendon collagens (b) obtained as in Fig. 8 were subjected to SDS-PAGE. Lanes 1, fractions 15; lane 2, fraction 16; lanes 3, fractions 19; and lanes 4, fractions 20. Std, molecular mass standards in kDa. $\alpha 1$, $\alpha 1$ chains of collagen; $\alpha 2$, $\alpha 2$ chains of collagen; β, β-chains of collagen; DF, dye front.

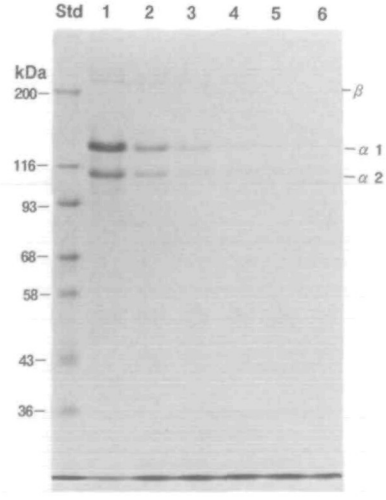

Fig. 10. **Pepsin digestion of control rat tail tendon collagen at 35°C.** Control rat tail tendon collagen, which had been preincubated with pepsin for 30 days at 30°C, was reincubated at 35°C. At the indicated times, aliquots of the incubation mixture were removed, neutralized with a NaOH solution, and then subjected to SDS-PAGE. The incubation times at 35°C were: lane 1, 0 h; lane 2, 0.5 h; lane 3, 1 h; lane 4, 2 h; lane 5, 3 h; and lane 6, 4 h. Std, molecular mass standards in kDa. $\alpha 1$, $\alpha 1$ chains of collagen; $\alpha 2$, $\alpha 2$ chains of collagen; β, β-chains of collagen.

below 30°C, was prepared as described under "MATERIALS AND METHODS," and then incubated with [^{14}C]ribose at 30°C. As shown in Fig. 11, the soluble collagen was found to be glycated as efficiently as, or more efficiently than, the fibrous collagen. Approximately 24 mol of ribose was incorporated into 1 mol of α-chains of soluble collagen in 30 days, while the level of glycation of fibrous collagen during the same incubation period at 37°C was approximately 16 mol ribose per mol α-chains (see Fig. 1A). The level of glycation of soluble collagen appeared to further increase on prolonged incubation with ribose (Fig. 11). When the glycated collagen was subjected to SDS-PAGE, it was found that the concentration of monomeric chains of the glycated soluble collagen did not change to a significant extent during incubation with ribose (Figs. 12 and 13), although fibrous collagen exhibited a rapid decrease in the concentrations of its monomeric chains during the incubation (see Figs. 1B and 2). It was found, however, that the monomeric chains of soluble collagen showed a gradual decrease in electrophoretic mobility with increasing incubation time (Fig. 12). These results indicated that the triple-helical regions of monomeric collagen do not undergo a significant extent of intramolecular or intermolecular cross-linking during incubation with ribose at 30°C. When the soluble collagen was incubated at 37°C, it immediately became insoluble in both the presence and absence of ribose due to the formation of gels of collagen fibrils (*29*). When the collagens thus incubated for 14 days at 37°C were boiled in an SDS solution, the collagen incubated without ribose was solubilized completely, while that incubated with ribose was solubilized partially (approximately 30%). On SDS-PAGE of these SDS extracts, the extract of the control incubation mixture was found to contain nearly identical concentrations of α1 and α2 chains as did the soluble collagen prior

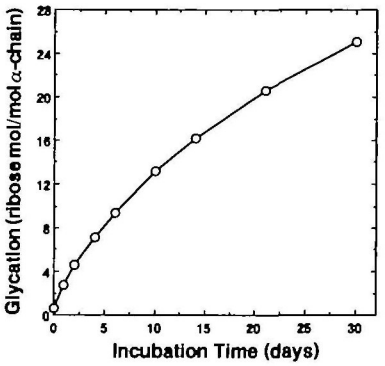

Fig. 11. **Time course of glycation of soluble collagen.** Soluble collagen, which consisted primarily of monomeric collagen, was prepared as described under "MATERIALS AND METHODS," and then incubated with 0.2 M [^{14}C]ribose at 30°C. On the indicated days, TCA was added to 20% to the mixtures, and then the radioactive ribose incorporated into the TCA-insoluble collagen was determined.

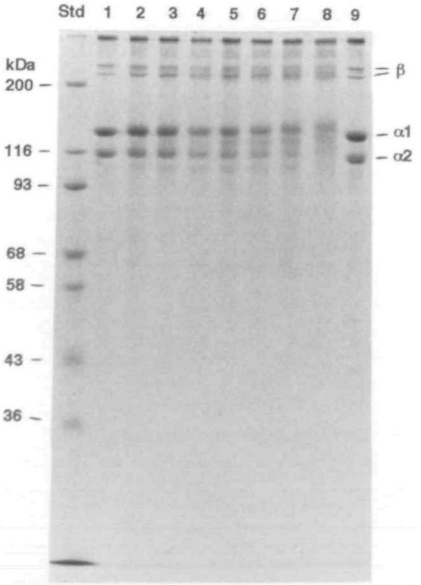

Fig. 12. **SDS-PAGE of glycated soluble collagen.** Soluble collagen was incubated with 0.2 M ribose for the indicated numbers of days and then subjected to SDS-PAGE. The numbers of days of incubation with ribose were: lane 1, 0 days; lane 2, 1 day; lane 3, 2 days; lane 4, 4 days; lane 5, 6 days; lane 6, 8 days; lane 7, 12 days; lane 8, 20 days; and lane 9, 0 days. Std, molecular mass standards in kDa. α1, α1 chains of collagen; α2, α2 chains of collagen; β, β-chains of collagen.

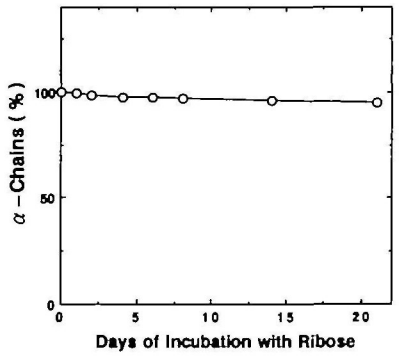

Fig. 13. **Time course of the change in the content of α-chains during glycation of soluble collagen.** Soluble collagen which had been glycated for the indicated numbers of days was subjected to SDS-PAGE, as in Fig. 12. The Coomassie Blue-stained α-chain regions were cut out from the gel, and the dye was quantitated as described under "MATERIALS AND METHODS."

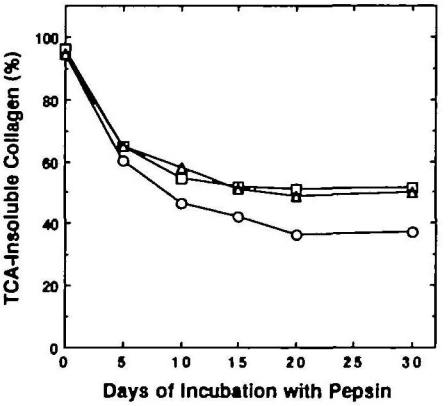

Fig. 14. **Time courses of degradation of control and glycated soluble collagen by pepsin.** Control soluble collagen (○) or soluble collagen glycated for 10 (□) or 20 days (△) was incubated with pepsin for the indicated numbers of days at 30°C and made to 20% with respect to TCA. The contents of collagen in the precipitates were determined.

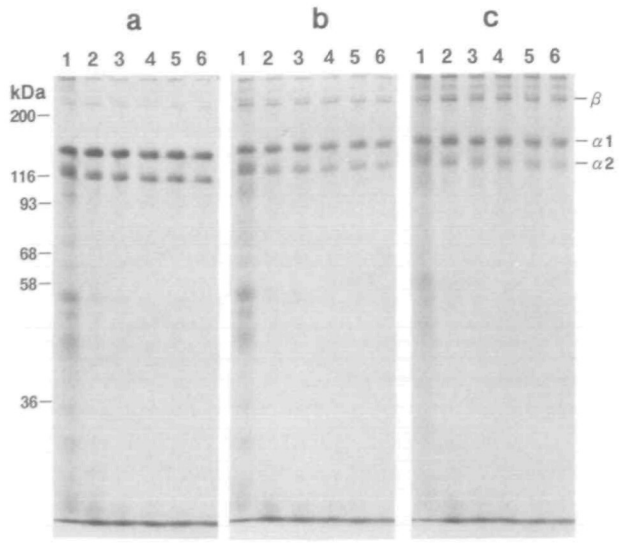

Fig. 15. **SDS-PAGE of pepsin digests of glycated soluble collagen.** Control soluble collagen (a) or soluble collagen glycated for 10 (b) or 20 days (c) was incubated with pepsin for the indicated numbers of days and then subjected to SDS-PAGE. The periods of incubation with pepsin were: lanes 1, 0 days; lanes 2, 5 days; lanes 3, 10 days; lanes 4, 15 days; lanes 5, 20 days; and lanes 6, 30 days. Std, molecular mass standards in kDa. $\alpha 1$, $\alpha 1$ chains of collagen; $\alpha 2$, $\alpha 2$ chains of collagen; β, β-chains of collagen.

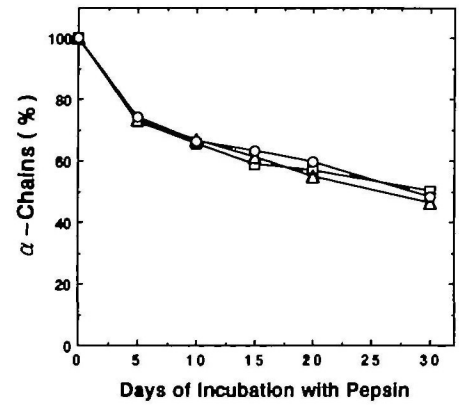

Fig. 16. **Time courses of the change in the content of α-chains of collagen during pepsin digestion of control and glycated soluble collagen.** Control soluble collagen (○) or soluble collagen glycated for 10 (□) or 20 days (△) was incubated with pepsin for the indicated numbers of days at 30°C and then subjected to SDS-PAGE as in Fig. 15, and then the Coomassie Blue bound to the α-chains was determined.

to incubation, while the extract of ribose-incubated collagen did not contain a significant concentration of monomeric or oligomeric chains of collagen (not shown), indicating that collagen fibrils reconstructed from the soluble collagen also undergo cross-linkings. Furthermore, when the collagen fibrils obtained on incubation at 37°C for 1 day in the absence of ribose were reincubated for 14 days with ribose at 30°C, they were found to undergo a similar extent of cross-linking to as observed for the fibrils incubated at 37°C, as judged from their resistance to boiling in an SDS solution, and from the complete loss of monomeric and oligomeric collagen chains. Similarly, when rat tail tendons were incubated with ribose at 30°C instead of 37°C, the collagen fibers were also found to undergo cross-linking at a rate similar to, but slightly lower than, that at 37°C (not shown). These results thus appeared to indicate that a fibrous state, but not an elevated temperature such as 37°C, is indispensable for the cross-linking of collagen.

Pepsin Digestion of Control and Glycated Soluble Collagens—When the soluble collagen that had been preincubated for 0, 10, or 20 days with ribose was incubated with pepsin at 30°C, all of the collagen was found to be gradually degraded with increasing incubation time, while the rates of degradation of the glycated collagens were slightly lower than that of the control collagen (Fig. 14). When the incubation mixtures were analyzed by SDS-PAGE, the triple-helical regions of control and glycated collagens were found to be degraded at nearly identical rates (Figs. 15 and 16). It was apparent that non-glycated control collagen and glycated, but not cross-linked, collagen exhibited nearly identical levels of stability against pepsin digestion at 30°C. The glycation-induced increase in proteolytic degradation of fibrous collagen by pepsin is thus likely to be due to the intermolecular cross-linking at the triple-helical regions rather than to non-cross-linking glycation.

DISCUSSION

It is generally accepted that glycation makes fibrous collagen resistant to pepsin digestion (*4, 15*). In the present study, we have shown that, under defined experimental conditions, glycation increases the proteolytic degradation of the triple-helical regions of collagen by pepsin rather than making them resistant to degradation. This difference appears to be due to the following reasons. Whereas the proteolytic degradation of control fibrous collagen proceeds in a well recognizable manner even at a low temperature, the degradation of glycated fibrous collagen proceeds in an unrecognizable manner or at an inappreciable rate at the temperatures examined previously. Namely, whereas limited proteolysis of the non-helical regions at a low temperature is effective in liberating the triple-helical regions from non-glycated fibrous collagen, it is ineffective in liberating the same regions from glycated fibrous collagen, since the intermolecular cross-links at the triple-helical regions interfere with the liberation of the same regions from the glycated collagen. It was found, however, that not only the triple-helical regions of glycated fibrous collagen but also the same regions of control collagen were both very stable against pepsin digestion at a low temperature, in spite that the triple-helical regions of control fibrous collagen were liberated efficiently, while those of glycated fibrous collagen were not. It is not known, however, whether glycation decreases or increases the proteolytic susceptibility of the non-helical regions to pepsin. Thus, previous studies do not appear to provide an answer to the question of whether glycation decreases or increases the proteolytic susceptibility of the triple-helical regions of collagen. It should be emphasized, however, that the triple-helical regions of glycated fibrous collagen become much less stable than those of control fibrous collagen against pepsin digestion only in a specific temperature range, *i.e.* a range close to, but below, the melting point of the strands, as discussed below.

The rate and extent of degradation of glycated fibrous

collagen by pepsin were found to increase greatly in a specific temperature range. Whereas most glycated collagen was resistant to pepsin digestion at 25°C, it was completely degraded into small peptides by pepsin at 30°C. The rate, but not the final extent, of its degradation appeared to increase further at 35°C or continue to increase up to and even above 35°C. Similarly, the rate and extent of degradation of the triple-helical strands of control collagen by pepsin increased greatly at 35°C, while the strands were fairly stable against pepsin digestion at 30°C. These temperature-dependent abrupt increases in the digestability of the collagens appear to arise from changes in the conformation or organization of the triple-helical strands of the collagen molecules rather than a change in the proteolytic activity of pepsin, since the magnitudes of the changes in the digestability appear to be much larger than those of the changes in the proteolytic activity at the respective temperatures. Although the changes in the digestability of both control and glycated fibrous collagens at 35°C appear to be induced by the unfolding of their triple-helical strands, it is not known through what type of conformational or organizational change the digestability of glycated fibrous collagen is increased at 30°C. Since the glycated, but not cross-linked, soluble collagen exhibited a similar level of stability against pepsin digestion at 30°C to that of control collagen, the increased degradation of glycated fibrous collagen at 30°C appears to be due to the presence of sugar-derived cross-links in the collagen. Furthermore, since the triple-helical strands did not appear to undergo intramolecular cross-linking, most of the cross-links produced in fibrous collagen are supposed to be intermolecular ones rather than intramolecular ones. These results appear to raise the possibility that the intermolecular cross-links in glycated fibrous collagen increase its proteolytic degradation by inducing local unfolding of the triple-helical strands at 30°C, thereby making them accessible to pepsin. Although the mechanism by which the intermolecular cross-links induce local unfolding of the triple-helical strands is not known, these cross-links are supposed to unfold the strands by interfering with the free thermal motions of the collagen molecules and their triple-helical strands. When one of the three strands in a collagen molecule is cross-linked with another strand in an adjacent collagen molecule, one or two of the non-cross-linked strands will temporarily unfold or separate from the cross-linked strands at the cross-linked sites, since the thermal motion of only the cross-linked strands is limited by the cross-links, *i.e.* that of the non-cross-linked ones is not. At such a relatively high temperature as close to the melting point or unfolding temperature of the native strands, the thermal motions of the collagen molecules and their individual strands may be large enough to induce unfolding of the modified strands.

It has been proposed that cross-linking of fibrous collagen by AGE is a primary mechanism that underlies the age-related and diabetes-accelerated decreases in its solubility and proteolytic digestability (*4, 5, 13, 15-17*). In the present study, we found that fibrous collagen that had been extensively cross-linked by a derivative of ribose underwent complete degradation on pepsin digestion at 30°C. Recently, we found that vascular tissues from middle-aged and old-aged humans, but not ones from young humans, contained a large fraction of fibrous collagen that was resistant to pepsin digestion at 30°C but was labile against pepsin digestion at 35°C (*31, 32*). These results suggest that fibrous collagen in living tissues undergoes a modification that is distinct from its modification by AGE produced by derivatives of ribose. These findings as well as the analytical results described in this report thus appear to reveal the differences between the changes occurring *in vivo* and *in vitro* of fibrous collagen, and to facilitate elucidation of the mechanism underlying the age-related and diabetes-accelerated changes in fibrous collagen.

A previous X-ray diffraction study demonstrated that glycation induces expansion of the unit cell area of collagen fibrils of rat tail tendons in a direction perpendicular to the axis of the rod-shaped molecules (*14*). The authors interpreted the present results as showing the expansion was brought about by the formation of intermolecular cross-links that acted to push the molecules apart. In the present study, we have shown that glycation induces unfolding of the triple-helical strands of collagen in an acidic medium. This change may also be able to expand the area of distribution of collagen chains within fibrils, although it is not known to what extent this change affects the X-ray diffraction pattern at neutral pH.

The stress-strain curve for a control tendon consists of three well-defined regions: a "toe" region, where initial extension occurs with little stress; a linear region, where stress is linearly proportional to strain; and a "yield and failure" region, where increasing extension results in breaking of the tendon (*33*). Bai *et al.* (*18*) have demonstrated that glycation induces changes in all three regions of the stress-strain curve, *i.e.* expansion of the toe and linear regions, and an increase in the ultimate stress of breaking. The expansion of the toe and linear regions is thus likely to be induced by the glycation-induced unfolding of the triple-helical strands, while the increased ultimate stress appears to be due to an increased number of intermolecular cross-links. Similarly, Yue *et al.* (*6*), and Kohn *et al.* (*13*) demonstrated previously that glycated collagen fibers exhibited an increased breaking time in a 7 M urea solution kept at 40 and 45°C, respectively, compared to control fibers. These previous studies now also appear to indicate that glycation increases the total mechanical stability of collagen fibers by increasing the stability of the interfibrillar and intermolecular interactions in the fibers rather than increasing the stability of intramolecular interactions among the triple-helical stands, since the triple-helical strands of both control and glycated fibrous collagens are supposed to dissociate under the conditions examined.

REFERENCES

1. Kohn, R.R. (1978) *Principles of Mammalian Aging*, 2nd ed., Prentice-Hall, Englewood Cliffs, New Jersey
2. Hamlin, C.R., Kohn, R.R., and Luschin, J.H. (1975) Apparent accelerated aging of human collagen in diabetes mellitus. *Diabetes* **24**, 902-904
3. Andreassen, T.T., Seyer-Hansen, K., and Bailey, A.J. (1981) Thermal stability, mechanical properties and reducible cross-links of rat tail tendon in experimental diabetes. *Biochim. Biophys. Acta* **677**, 313-317
4. Schnider, S.L. and Kohn, R.R. (1981) Effects of age and diabetes mellitus on the solubility and nonenzymatic glucosylation of human skin collagen. *J. Clin. Invest.* **67**, 1630-1635
5. Schnider, S.L. and Kohn, R.R. (1982) Effects of age and diabetes mellitus on the solubility of collagen from human skin, tracheal

cartilage and dura matter. *Exp. Gerontol.* **17**, 185-194
6. Yue, D.K., McLennan, S., Delbridge, L., Handelsman, D.J., Reeve, T., and Turtle, J.R. (1983) The thermal stability of collagen in diabetic rats: Correlation with severity of diabetes and non-enzymatic glycosylation. *Diabetologia* **24**, 282-285
7. Sell, D.R. and Monnier, V.M. (1989) Structure elucidation of a senescence cross-link from human extracellular matrix: Implication of pentoses in the aging process. *J. Biol. Chem.* **264**, 21597-21602
8. Grandhee, S.K. and Monnier, V.M. (1991) Mechanism of formation of the Maillard protein cross-link pentosidine: Glucose, fructose, and ascorbate as pentosidine precursors. *J. Biol. Chem.* **266**, 11649-11653
9. Dyer, D.G., Blackledge, J.A., Thorpe, S.R., and Baynes, J.W. (1991) Formation of pentosidine during nonenzymatic browning of proteins by glucose: Identification of glucose and other carbohydrates as possible precursors of pentosidine *in vivo*. *J. Biol. Chem.* **266**, 11654-11660
10. Brownlee, M. (1995) Advanced protein glycosylation in diabetes and aging. *Annu. Rev. Med.* **46**, 223-234
11. Bailey, A., Sims, T.J., Avery, N.C., and Halligan, E.P. (1995) Non-enzymatic glycation of fibrous collagen: Reaction products of glucose and ribose. *Biochem. J.* **305**, 385-390
12. Bunn, H.F. and Higgins, P.J. (1981) Reaction of monosaccharides with proteins: Possible evolutionary significance. *Science* **213**, 222-224
13. Kohn, R.R., Cerami, A., and Monnier, V.M. (1984) Collagen aging in vitro by nonenzymatic glycosylation and browning. *Diabetes* **33**, 57-59
14. Tanaka, S., Avigad, G., Brodsky, B., and Eikenberry, E.F. (1988) Glycation induces expansion of the molecular packing of collagen. *J. Mol. Biol.* **203**, 495-505
15. Tanaka, S., Avigad, G., Eikenberry, E.F., and Brodsky, B. (1988) Isolation and partial characterization of collagen chains dimerized by sugar-derived cross-links. *J. Biol. Chem.* **263**, 17650-17657
16. Kent, M.J.C., Light, N.D., and Bailey, A.J. (1985) Evidence for glucose-mediated covalent cross-linking of collagen after glycosylation in vitro. *Biochem. J.* **225**, 745-752
17. Fu, M.-X., Wells-Knecht, K.J., Blackledge, J.A., Lyons, T.J., Thorpe, S.R., and Baynes, J.W. (1994) Glycation, glycoxidation, and cross-linking of collagen by glucose: Kinetics, mechanism, and inhibition of late stages of the Maillard reaction. *Diabetes* **43**, 676-683
18. Bai, P., Phua, K., Hardt, T., Cernadas, M., and Brodsky, B. (1992) Glycation alters collagen fibril organization. *Connect. Tissue Res.* **28**, 1-12
19. Bornstein, P. and Traub, W. (1979) The chemistry and biology of collagen in *The Proteins*, 3rd ed. (Neurath, H. and Hill, R.L., eds.) Vol. 4, pp. 411-632, Academic Press, New York
20. van der Rest, M. and Garrone, R. (1991) Collagen family of proteins. *FASEB J.* **5**, 2814-2823
21. Eyre, D.R., Paz, M.A., and Gallop, P.M. (1984) Cross-linking in collagen and elastin. *Annu. Rev. Biochem.* **53**, 717-748
22. Reiser, K., McCormick, R.J., and Rucker, R.B. (1992) Enzymatic and nonenzymatic cross-linking of collagen and elastin. *FASEB J.* **6**, 2439-2449
23. Miller, E.J. and Rhodes, R.K. (1982) Preparation and characterization of the different types of collagen in *Methods in Enzymolgy* (Cunningham, L.W. and Frederiksen, D.W., eds.) Vol. 82, pp. 33-64, Academic Press, New York
24. Berg, R.A. (1982) Determination of 3- and 4-hydroxyproline in *Methods in Enzymology* (Cunningham, L.W. and Frederiksen, D.W., eds.) Vol. 82, pp. 382-398, Academic Press, New York
25. Matsumura, S., Higashino, H., Takashima, T., Ikeda, N., Fujitani, N., and Muguruma, M. (1992) Comparative studies on the extractability of collagen from aortas of stroke-prone spontaneously hypertensive and normotensive rats. *Biochim. Biophys. Acta* **1139**, 1-8
26. Horgan, D.J., King, N.L., Kurth, L.B., and Kuypers, R. (1990) Collagen crosslinks and their relationship to the thermal properties of calf tendons. *Arch. Biochem. Biophys.* **281**, 21-26
27. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
28. Fenner, C., Traut, R.R., Mason, D.T., and Wikman-Koffelt, J. (1975) Quantification of Coomassie Blue stained proteins in polyacrylamide gels based on analyses of eluted dye. *Anal. Biochem.* **63**, 595-602
29. Oegema, T.R., Jr., Laidlaw, J., Hascall, V.C., and Dziewiatkowski, D.D. (1975) The effect of proteoglycans on the formation of fibrils from collagen solutions. *Arch. Biochem. Biophys.* **170**, 698-709
30. Dick, Y.P. and Nordwig, A. (1966) Effect of pH on the stability of the collagen fold. *Arch. Biochem. Biophys.* **117**, 466-468
31. Matsumura, S., Kawazoye, S., Tian, S.-F., Takashima, T., Sunaga, T., Toda, S., Fujitani, N., Higashino, H., and Suzuki, A. (1995) Biochemical characterization of the fibers of collagen and elastin in the aortic wall of SHRSP. *Clin. Exp. Pharmacol. Physiol.* **22**, Suppl. **1**, S136-S138
32. Matsumura, S., Kawazoye, S., and Tian, S.-F. (1995) Aging of extracellular matrix of arterial wall. *Connect. Tissue* **27**, 213-220
33. Kastelic, J. and Baer, E. (1980) Deformation of tendon collagen. *Symp. Soc. Exp. Biol.* **34**, 397-435