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Cross-linking and fluorescence changes of collagen by glycation and oxidation

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The non-enzymatic glucosylation of collagen in vivo and in vitro produces blue-fluorescent cross-links very slowly. The mechanism of their formation is unknown. We investigated the role of oxidation in glycation. When native fluorescent collagen from old-rat tail tendon and its CNBr peptides were oxidized by chemically generated singlet oxygen, cross-linking occurred immediately, and the cross-linked products showed an increased blue fluorescence. Further cross-linking and development of blue fluorescence also were accelerated by singlet oxygen when oxidizing in vitro glucosylated collagen CNBr peptides. It was noted that the blue fluorescence developed at the expense of a near-UV fluorescence. This near-UV fluorophore, which is also present in native collagen, was found to be produced by the in vitro glucosylation of collagen and during the cross-linking by glucosylation was slowly converted to the blue fluorophore. These changes indicate the autoxidation of near-UV fluorescent intermediates to blue fluorescent cross-links during glucosylation. Non-enzymatic fructosylation, which occurs in vivo in certain proteins, was more effective than glucosylation in forming fluorophores and cross-links with collagen in vitro. Fructosylated fluorophores were found different from glucosylated products in their oxidation reactivities with singlet oxygen.

Introduction

Glycation (non-enzymatic glycosylation), cross-linking and fluorophore formation of collagen occur both in vivo and in vitro. In vivo, native collagen becomes more blue-fluorescent [1–4] and glycated with aging [5] and diabetes [6]. Recently, dimers (β-components) of two collagen α-chains, cross-linked as a result of glycation, have been found in pepsin digests of tail tendons from older rats [7]. Although glycated collagens, including these dimers, are fluorescent, the fluorescence characteristics and distribution in CNBr peptides have not been investigated. Our recent studies on environment-related changes in collagen showed that ozone oxidation or UV (300 nm) photooxidation of fluorescent CNBr peptides from insoluble collagen of old-rat tail tendon increases their cross-linking and causes changes in fluorescence [8]. A blue fluorescence at 430 nm (excited at 350 nm) in cross-linked peptides increased at the expense of a near-UV fluorescence at 360/370 nm (excited at 300 nm) in non-cross-linked peptides. This novel near-UV fluorescence had been detected originally in monomeric collagen α-chains and was found susceptible to ozone and UV (300 nm) radiation [9].

In addition to the cross-linking, ozone also degraded the peptides, and this was attributed to the effect of hydroxyl radicals formed in the decomposition of ozone in water. On the other hand, UV (300 nm) irradiation, possibly forming singlet oxygen photochemically, did not degrade the collagen peptides [8]. The present study reports the effects of chemically generated singlet oxygen upon the structure and fluorescence properties of type I collagen and its CNBr peptides. Singlet oxygen can be generated by chemical and photochemical reactions [10] and is present in polluted atmospheres [11,12]. For comparison, the effect of hydroxyl radicals was also studied.

Incubation of collagen with glucose in solutions results in non-enzymatic binding of glucose to collagen. This in vitro glucosylation also increases blue fluorescence [13] and gives rise to covalent cross-linking [14]. The blue fluorescent compounds could play a role in cross-linking [15,16]. The study of young-rat tail tendon in solutions containing D-ribose showed that cross-linked α-chain dimers are strongly blue-fluorescent [7]. Glycation and cross-link formation by D-ribose are far more effective than glucose, but closely resemble those

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obtained with glucose. In this work, fluorescence changes and cross-linking of CNBr peptides from in vitro glucosylated collagen by oxidation were investigated and compared with those from in vivo glucosylated native collagen. Furthermore, frucosylated collagen was also studied. Although aldoses generally react with protein more rapidly than do ketoses, the percent carbonyl form of fructose (0.7%) is much higher compared with that of glucose (0.002%) [17].

Materials and Methods

Collagen and its CNBr peptides

Acid-soluble and -insoluble collagens were prepared from old-rat (about 2 years old) tail tendon as previously reported [3,8,9]. Digestion of lyophilized insoluble collagen (100 mg) with CNBr (600 mg) was carried out in 70% formic acid (10 ml) at 30°C for 6 h. The CNBr digests were diluted with distilled water (100 ml), lyophilized and dissolved (8 mg/ml) in 0.1% SDS, 0.1 M borate buffer (pH 8.2). After heating at 60°C, the digests were allowed to stand at room temperature for at least 2 days. Then, 0.1% SDS-soluble peptides were separated from 0.1% SDS-insoluble peptides and used for oxidation.

Oxidation

Acid-soluble collagen and 0.1% SDS-soluble CNBr peptides from insoluble collagen were oxidized by singlet oxygen. Acid-soluble collagen dissolved in 0.05 M acetic acid (2.5 mg/ml) was mixed (1:1, v/v) with 0.2 M phosphate buffer (pH 7.5) and immediately oxidized by singlet oxygen. Singlet oxygen was generated by the chemical system: 10 mM NaOCl/10 mM H_2O_2. Sodium hypochlorite NaOCl was added to CNBr peptide solution or acid-soluble collagen solution to which H_2O_2 had been added. Singlet oxygen-oxidized collagen was treated with 5% mercaptoethanol for 2 days to reduce oxidized methionine groups, washed with distilled water, lyophilized, and digested with CNBr. The CNBr digests were lyophilized and dissolved in 0.1% SDS buffer solution. CNBr peptides from insoluble collagen were also oxidized by hydroxyl radicals. Hydroxyl radicals were produced from 0.1 mM $CuCl_2$/10 mM ascorbate by adding ascorbate to CNBr peptide solution containing $CuCl_2$.

Glycation

Insoluble collagen (100 mg) was incubated with 0.5 M glucose or fructose (both from Sigma) in 10 ml of 0.2 M phosphate buffer (pH 7.5) containing 0.2% sodium azide. After incubation for 4–9 days at 37°C, insoluble collagen was separated from the buffer solution, thoroughly washed with distilled water, and lyophilized before CNBr digestion. As with collagen CNBr peptides, 0.1% SDS-soluble CNBr peptides from glucosylated/fructosylated collagens were oxidized by singlet oxygen.

Separation of CNBr peptides

Before and after oxidation, the CNBr peptides were separated by gel filtration or gel electrophoresis. For gel filtration, a column (1.3 × 200 cm) of Sephacryl S-200 Superfine (Pharmacia) was used in 0.1% SDS, 0.1 M borate buffer (pH 8.2). For gel electrophoresis, a SDS-polyacrylamide (7.5%) gel was employed. Fluorescence spectra (uncorrected) and relative fluorescence intensities at 370 nm (excited at 300 nm) and at 430 nm (excited at 350 nm) were measured using a Perkin-Elmer 650-10S fluorescence spectrophotometer, while absorbance at 230 nm a Shimadzu UV-160 spectrophotometer.

Results

Oxidation of collagen CNBr peptides by singlet oxygen or hydroxyl radical

Fig. 1A, curve 1 (230 nm absorbance changes) shows a gel-filtration profile of 0.1% SDS-soluble CNBr digests from fluorescent insoluble collagen of old-rat tail tendon. A peak migrating at the void volume (polymeric

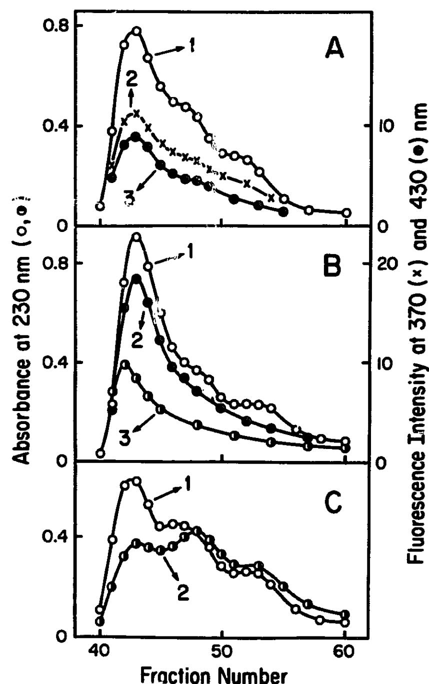

Fig. 1. Gel-filtration of collagen CNBr peptides before (A) and after oxidation by singlet oxygen (B) or hydroxyl radical (C). SDS (0.1%)-soluble peptides from insoluble collagen CNBr digests (2.4 mg/3 ml) were fractionated (2.3–2.4 ml/8 min) by gel-filtration on Sephacryl S-200. (A) Control; curve 1 (230 nm absorbance); curve 2 (370 nm relative fluorescence intensity, excited at 300 nm); curve 3 (430 nm fluorescence, excited at 350 nm). (B) After treatment with 10 mM H_2O_2/10 mM NaOCl for 5 min: curve 1 (230 nm absorbance); curve 2 (430 nm fluorescence). (C) After treatment with 0.1 mM $CuCl_2$/10 mM ascorbate: curve 1, 10 min; curve, 2, 40 h incubation (230 nm absorbance for both curves 1 and 2). Singlet oxygen-oxidized acid-soluble collagen (30 mg) treated with 5% mercaptoethanol was partially digested by CNBr. CNBr digests in 0.1% SDS buffer were fractionated: (B) curve 3 (230 nm absorbance).

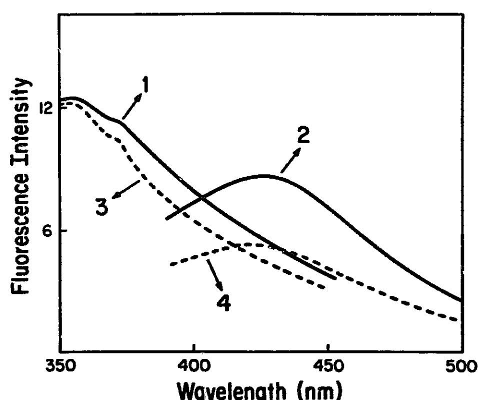

Fig. 2. Fluorescence spectra of CNBr peptide fractions from collagen and glucosylated (6 days) collagen. The spectra (relative intensity) of fraction 43 in Fig. 1A (spectra 1 and 2) and fraction 52 in fig. 3A (spectra 3 and 4) were measured. Spectra 1 and 3 were excited at 300 nm, spectra 2 and 4 at 350 nm.

peptides) was followed by two shoulders consisting mainly of the larger peptides α2CB3, 4, 5 (29–31 kDa) and α1CB7, 8 (24–25 kDa). The 370 and 430 nm fluorescence intensities, excited at 300 and 350 nm, respectively, of these peptides are shown in Fig. 1A, curves 2 and 3. The amount of cross-linked peptides, dissolved in 0.1% SDS-buffer solution, was relatively higher and their fluorescence spectra in peak fraction 43 (Fig. 1A) are depicted in Fig. 2, spectra 1 and 2.

As shown in Fig. 1B, curve 1, the exposure of collagen CNBr digests to singlet oxygen from 10 mM each of H_2O_2 and NaOCl in 0.1 M borate (pH 8.2) increased the amount of polymerized peptides (first peak), also migrating at the void volume (> 100–250 kDa), at the expense of peptides in the two shoulders. At the same time, the 430 nm fluorescence of polymeric peptides markedly increased (Fig. 1B, curve 2). SDS-gel electrophoresis showed that the oxidized peptides could not enter a 7.5% polyacrylamide gel. When acid-soluble collagen was exposed to singlet oxygen in 0.1 M phosphate buffer (pH 7.5), it was immediately coagulated. The oxidized collagen was treated with 5% mercaptoethanol for 2 days, but only partially solubilized by CNBr. The resulting CNBr peptides in 0.1% SDS buffer, when gel-filtered, gave rise to only cross-linked peptides (Fig. 1B, curve 3) with enhanced 430 nm fluorescence. The 430 nm fluorescence intensity of oxidized acid-soluble collagen peptides at fraction 42 (230 nm absorbance: 0.39) was 27% higher than that of non-oxidized acid-soluble collagen peptides (230 nm absorbance: 0.83). The 430 nm fluorescence of the latter was about one half of that of insoluble collagen peptides, shown in Fig. 1A, curve 3.

In contrast to singlet oxygen, hydroxyl radicals from 0.1 mM $CuCl_2$ and 10 mM ascorbate, degraded CNBr peptides, most prominently polymeric peptides (Fig. 1C, curves 1 and 2).

Oxidation of glucosylated collagen CNBr peptides by singlet oxygen

Insoluble collagen that had been non-enzymatically glucosylated with 0.5 M glucose for 4–9 days at 37°C was solubilized by CNBr within 30 min at 30°C. The gel-filtration pattern of 0.1% SDS-soluble CNBr peptides from glucosylated (6 days) collagen showed the increased formation of cross-linked peptides (first peak in Fig. 3A, curve 1) compared with non-glucosylated collagen peptides (first peak in Fig. 1A, curve 1). Increase of the second and third peaks could be due to the increased solubility of glucosylated CNBr peptides in 0.1% SDS buffer solution. The intensity of 430 nm fluorescence (excited at 350 nm) in cross-linked peptides was more than doubled by glucosylation (first peak in Fig. 3A, curve 3). This increase is compatible with the formation of blue-fluorescent glycation-mediated cross-links. Previous in vitro glucosylation studies on acid-soluble collagen indicated that glucosylation could occur with all major CNBr peptides [18] or predominantly in α2CB4 and α1CB6 [19]. Similar studies on collagen fibrils [14,15] or tendons [7,14] showed increasing amounts of high molecular mass cross-linked peptides. All three fluorescent dimers, β11, β12 and β22, obtained from ribose-treated young-rat tail tendon also showed a large amount of high molecular mass CNBr peptides [7]. In this study of insoluble collagen, collagen-linked fluorophores were observed in the major polymeric and larger peptides.

The most remarkable change by in vitro glucosylation that had not been recognized before was a marked increase of near-UV fluorescence at 370 nm (excited at

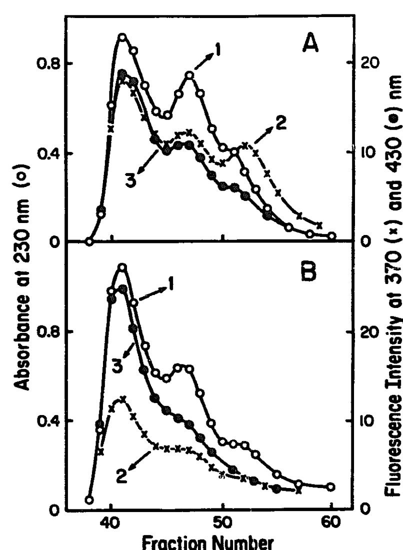

Fig. 3. Gel filtration of glucosylated collagen CNBr peptides before (A) and after (B) oxidation by singlet oxygen. SDS (0.1%)-soluble CNBr peptides from glucosylated (6 days) insoluble collagen were fractionated (2.4–2.5 ml/8 min) by gel-filtration on Sephacryl S-200. (A) Control. (B) After treatment with 10 mM H_2O_2/10 mM NaOCl for 5 min: curve 1 (230 nm absorbance), curve 2 (370 nm fluorescence, excited at 300 nm), curve 3 (430 nm fluorescence, excited at 350 nm).

300 nm) in the intermediate stages (4–6 days) of glucosylation. This can be seen clearly in non-cross-linked peptides at fractions 50–54 from 6 days' glucosylated collagen (third peak in Fig. 3A, curve 2). The fluorescence spectra of fraction 52 (Fig. 3A) from in vitro glucosylated (6 days) collagen (Fig. 2, spectra 3 and 4) are compared in Fig. 2 with those of fraction 43 (Fig. 1A) from in vivo glucosylated native collagen (Fig. 2, spectra 1 and 2). Spectra 1 and 3 were excited at 300 nm, while spectra 2 and 4 at 350 nm. Both in vivo and in vitro glucosylated collagen peptides exhibited the same spectra. The identical spectral structure of near-UV fluorescence (excited at 300 nm) with two small peaks at about 360 and 370 nm in spectra 1 and 3 is especially noteworthy. These findings also support the origin of the fluorescence of native collagen from in vivo glucosylated residues. Longer in vitro glucosylated (9 days) collagen revealed a further increase of 430 nm fluorescence in polymerized peptides with a decrease of 370 nm fluorescence in non-polymeric peptides. These changes indicate that the 370 nm fluorophore is an intermediate precursor to the 430 nm fluorophore in glucosylation.

This slow transition from 370 to 430 nm, occurring during glucosylation, was found to be accelerated by oxidation with singlet oxygen. When CNBr peptides from in vitro glucosylated (6 days) collagen were oxidized by singlet oxygen, the immediate increase of polymerized peptides was accompanied by the increase of 430 nm fluorescence (Fig. 3B, curves 1 and 3). These increases occurred at the expense of non-polymeric peptides and their 370 nm fluorescence from glucosylated residues (Fig. 3B, curve 2). These results are consistent with the oxidation of 370 nm fluorophores to 430 nm fluorescent cross-links. Accordingly, the 430 nm blue fluorophores of collagen could be formed gradually by the autoxidation of 370 nm fluorescent intermediates during in vivo and in vitro glucosylation.

Oxidation of fructosylated collagen CNBr peptides by singlet oxygen

A similar blue fluorescence developed with greater intensity for fructose than glucose. This fluorescence change was accompanied by the markedly increased formation of cross-linked oligomers. Fructose was much more effective than glucose in inducing cross-linking. Fig. 4A, curve 1 shows a gel-filtration profile of 0.1% SDS-soluble CNBr peptides from insoluble collagen incubated with 0.5 M fructose for 4 days. Fructosylated (4 or 9 days) collagen, not easily solubilized by CNBr, took 1.5–2 h at 30°C for solubilization to occur. The 370 and 430 nm fluorescence intensities are shown in Fig. 4A, curves 2 and 3 respectively. When collagen was incubated with 0.5 M fructose for 9 days, the 230 nm absorbance of polymeric peptides reached 1.6 compared with 1.15 for incubation for 4 days shown in Fig. 4A, curve 1, while the 430 nm fluorescence jumped 3-fold from 50 to 160.

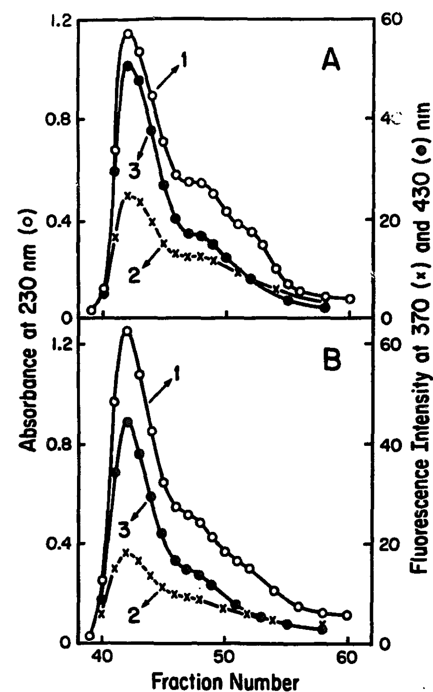

Fig. 4. Gel-filtration of fructosylated collagen CNBr peptides before (A) and after (B) oxidation by singlet oxygen. SDS (0.1%)-soluble CNBr peptides from fructosylated (4 days) insoluble collagen were fractionated (2.4 ml/8 min) by gel filtration on Sephacryl S-200. (A) Control. (B) After treatment with 10 mM H_2O_2/10 mM NaOCl for 5 min: curve 1 (230 nm absorbance), curve 2 (370 nm fluorescence, excited at 300 nm), curve 3 (430 nm fluorescence, excited at 350 nm).

When fructosylated (4 days) collagen CNBr peptides were oxidized by singlet oxygen, a small increase of polymeric peptides (Fig. 4B, curve 1) was not accompanied by the increase of 430 nm fluorescence, but rather by its decrease (Fig. 4B, curve 3). This could be due to the oxidation without cross-link cleavage of fructose-induced 430 nm fluorophores by singlet oxygen. This change was seen more in the increased formation of these fluorophores in CNBr peptides from fructosylated (9 days) collagen. Upon oxidation by singlet oxygen, in this case, there was only a slight increase in polymerized peptides, but the 430 nm fluorescence intensity dropped to 70% of the original intensity from 160 to 110. These results indicate a structural difference between fructosylated and glucosylated fluorophores. Fructose is mainly attached to protein via carbon 2, whereas glucose via carbon 1. Because of the strong intensity of 430 nm fluorescence and some energy transfer, the peak of near-UV fluorescence was not observed at 360–370 nm, but shifted to a longer wavelength around 400 nm, in fructosylated collagen CNBr peptides. Nevertheless, the fluorescence intensity, excited at 300 nm, was recorded at 370 nm (Fig. 4, curve 2).

Discussion

It was reported previously that singlet oxygen inhibits collagen fibril formation [20]. The present study reveals that the oxidation of native collagen and its CNBr peptides by singlet oxygen immediately forms blue-fluorescent cross-links. Singlet oxygen did not degrade collagen CNBr peptides just like UV (300 nm) irradiation, though hydroxyl radicals did degrade them. Therefore, oxidation by singlet oxygen was conveniently used to produce cross-links in this work. In our previous studies on the formation of cross-links by ozone oxidation or UV (300 nm) photooxidation, the origin of cross-links was not determined [8]. By comparing fluorescence changes and cross-linking occurring during in vitro glucosylation and in the oxidation of native and in vitro glucosylated collagen by singlet oxygen, this work concludes that cross-linking and related fluorescence changes are all glucosylation-mediated. Both blue and near-UV fluorophores, as well as cross-links, are originated from in vivo and in vitro glucosylated products.

Native collagen possesses in vivo glucosylated amino groups in both lysine and hydroxylysine residues [21,22]. Both in vivo and in vitro, these reactions form aldimine/ketoamine products and possibly a series of other products such as 3-deoxyglucosone/pyrraline [23]. Stable cross-links may arise from the condensation of two products [15,23] or, under certain conditions, one product on one chain with a free amino group on another chain [24]. The early attachment of glucose to collagen produces near-UV fluorescent products which are not involved in cross-linking. Whether or not the products such as ketoamine/pyrraline can be fluorescent remains to be further investigated. Aliphatic ketones and aldehydes are known to absorb around 300–320 nm and fluoresce [25]. These products are converted to blue-fluorescent cross-links gradually during in vivo/in vitro glucosylation. This conversion was effectively accelerated by oxidation with singlet oxygen. These results indicate the possible involvement of autoxidation in glucosylation. Hicks et al. [26] have recently reported an increase in cross-linking (as determined by increases in the thermal rupture time of the tendons) of normal and non-enzymatically glucosylated rat tail tendon collagen, after treatment with decomposing lipid hydroperoxides. The results of our study provide direct evidence for enhanced cross-linking induced by oxidation.

Recently, the contribution of glucose autoxidation to glucosylation has been suggested [27–29]. Free radicals may play a role in oxidation and glucosylation. Superoxide ($^{\cdot}O_2^-$) and H_2O_2 are generated during the autoxidation of a variety of reductants (AH_2). Ascorbate [30] and monosaccharides [31] undergo autoxidation, especially when transition metal ions (Cu^{2+} or Fe^{3+}) are present. Free radical intermediates ($AH^{\cdot}$), as well as superoxide radicals, could occur in glucosylated proteins during their autoxidation [32–34]. Transition metal ions catalyze the reactions of $^{\cdot}O_2^-$ or $AH^{\cdot}$ with H_2O_2 to produce hydroxyl ions ($^{\cdot}OH$). Thus, ascorbate autoxidation induces cross-linking or degradation of collagen, depending upon the conditions used [35], while cross-linking [36] or fragmentation [29] of serum albumin by glucose were also reported. The degradation of collagen by glucosylation, however, was not detected.

The chemical nature of fluorescent cross-linking compounds in non-enzymatically glucosylated proteins has not been elucidated. A blue-fluorescent compound, 2-(2-furoyl-4(5)-2-furanyl)-1*H*-imidazole (FFI) was isolated from the acid hydrolysate of bovine serum albumin and poly-L-lysine which had been incubated with glucose. It was proposed that this compound represents a fluorescent cross-link [15]. But recent studies do not support the proposition that FFI is an advanced glucosylation end product or a protein cross-link [37,38]. It is suggested that FFI is formed from ammonia and furosine [39] which are by-products of the acid-hydrolyzed Amadori product of glucosylated lysine.

In vivo, human lens proteins react with endogenous fructose and undergo non-enzymatic fructosylation [40]. In some organs such as ocular lenses, fructose is biosynthesized by oxidation of sorbitol. In diabetic subjects, concentrations of fructose often approach and sometimes exceed those of glucose in ocular lenses [41]. Fructosylation also may lead to cross-linking which contributes to diabetic complications. In vitro, fructose reacts with bovine serum albumin, producing a new fluorescence at 410 nm when excited at 350 nm [42]. The fluorescence of bovine serum albumin with glucose was about one-tenth of that with fructose. Ribonuclease A developed a fluorescence at 405 nm (excited at 330 nm) that is about 4-times stronger for fructose than for glucose [40]. Fructose was 10-times as effective as glucose in inducing cross-linking. Similar changes occurred with collagen, but a structural difference between fructosylated fluorophores and glucosylated products was reflected by their distinction in oxidation reactivities. These still poorly understood reactions are important for the pathologic complications of diabetes and remain to be further elucidated.

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