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Change in the Amount of ε-hexosyllysine, UV Absorbance, and Fluorescence of Collagen With Age in Different Animal Species

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Skin and aorta collagen specimens of Wistar rats, white mice, beagle dogs, cats, horses, and human necropsies of different ages were examined with respect to the content of glycated products. The data presented show that (a) glycation and accumulation of the chromophore(s) are comparable in collagen samples from different species of comparable age; (b) glycation and pigmented accumulation increase markedly during the first 5–10 years of age; (c) the extent of glycation is different in different tissues (in particular, glycation of aortal collagen is about twice that of skin collagen); and (d) collagen pigmentation as followed by fluorescence is comparable in aortal and skin collagen (except below 10 years); pigmentation measured by absorbance at 350 nm is, on the contrary, lower in aortal than in skin collagen. Based on the assumption of constant blood glucose level during the life span, it appears feasible to conclude that the degree of nonenzymatic collagen glycation reflects the time period for which the protein was exposed to the action of sugars. This period, because of increased cross-linking, is likely to be extended in older animals. Other factors, such as differences in collagen turnover between different tissues and the intensity of the removal process of the glycated products, should be taken into consideration as well.

IT appears well established that free amino groups can react in vivo with aldehydic sugars, particularly with glucose and glucose-6-phosphate via the Amadori rearrangement, and subsequent condensation reactions to form colored and fluorescent pigments, the nature of which is a matter of discussion. Some indications can be found in the literature about an age-related increase of fluorescence and brown pigment formation in long-lived proteins such as collagen and lens crystallins. The resistance of such proteins to enzymatic cleavage is indicative of the cross-linking nature of these advanced glycosylation end products (AGEs). Indeed, as demonstrated by Tanaka et al. (1988), collagen preparations incubated in vitro with ribose exhibit a considerably higher proportion of polymerized collagen α-chains in comparison to controls. Nonenzymatic binding of glucose to collagen results in decreased solubility of the protein, increased fluorescence, and changes in mechanical properties similar to changes observed in aging.

Although most evident in long-lived proteins such as collagen (Rosenberg et al., 1979) or lens crystallin (Cerami et al., 1979), products of nonenzymatic glycation have been also detected in hemoglobin (Bunn et al., 1978), erythrocyte membrane protein (Bailey et al., 1976), serum albumin (Day et al., 1979), and other serum proteins. Most of these results were obtained by boronate affinity chromatography; that is, in the glycation products the vicinal diol structure must have been preserved and, consequently, the results refer mainly to the early glycation products. Nonenzymatic glycation is likely to reflect the ubiquitous presence of glucose as the key compound of energetic metabolism in the animal's body. Further understanding of these reactions may elucidate the well-known relation between the intensity of metabolism and life span of a particular animal species. Recently the role of advanced glycosylation end products in aging was further stressed by demonstrating their presence in nucleic acids and nucleoprotein complexes (Bucala et al., 1984). It was shown that here the glucose reacts first with a lysine residue to form a reactive intermediate that can further react with single- or double-stranded DNAs under the formation of mixed (covalently) bound polymers (see Cerami et al., 1988).

The amount of AGEs formed in various body proteins is far lower than would be anticipated from the in vitro studies. This disparity induced a search for a mechanism capable of removing AGEs. Recently it has been demonstrated that macrophages display a high-affinity receptor that mediates uptake and degradation of AGE proteins (Vlassara et al., 1985). The macrophage receptor for AGE proteins appears unique in that it represents the first receptor described so far capable of recognizing an in vivo occurring ligand formed in a time-dependent manner.

A number of methods are available for quantitation of protein glycation. Ion exchange chromatography (Trivelli et al., 1971) or boronate affinity chromatography (Mallia et al., 1981) perhaps should be mentioned first. The determination of glycated products by means of assaying furosine (the acid hydrolysis product of glycated proteins) after reversed phase separation (C_{18}) represents another possible way (Schleicher and Wieland, 1981). Alterations introduced by glycation in proteins also can be revealed by electromigration methods, i.e., agar gel electrophoresis (Forrest et al., 1988) and isoelectric focusing (Mortensen, 1980). Currently colorimetric determination of protein glycation appears to be the most popular method. In this respect the first one to be mentioned is the thiobarbituric acid test (McFarland et al., 1979), but there are additional possibilities, such as the fructosamine test (Johnson et al., 1982) or the fluorometric

detection of formaldehyde from the periodate oxidation of glycated proteins (Gallop et al., 1981). In our study we used the thiobarbituric acid technique and boronate affinity chromatography.

The aim of the present investigation was to show how the concentration of both the early glycation product (ε-hexosyllysine) and the chromophore(s) exhibiting an absorbance at 350 nm and fluorescence at $370_{ex}/440_{em}$ nm are changed with age in different animal species and tissues, assuming that the chromophore can be classified as part of an AGE-modified protein.

Materials and Methods

Material sources. — Skin and aorta samples were obtained from Wistar rats, white mice, beagle dogs, and cats grown in the Department of the Biological Experimental Models of the Institute of Physiology, Prague, Czechoslovakia, with a precise record of their birthdate. Samples of horse material were obtained from a local slaughterhouse. Samples of human material originated from necropsies, obtained from a local hospital either from people killed during traffic accidents (aged 20–22 years) or from people who had died from heart attack (aged 73–75 years) without any signs of atherosclerosis and without any previous record of hypertension.

Tissues investigated. — Segments of thoracic aorta (2–4 cm long) or samples of dorsal skin (4 × 4 cm) were excised, freed from extraneous tissues, flushed with isotonic saline, and defatted by successive immersions in acetone and ether for 18 hours, dried for 24 hours in vacuo, and weighed.

Solubilization of collagen. — Collagen was solubilized by autoclaving each aortal and skin specimen in 5 ml distilled water for 18 hours at 103.5 N/m² and decanting the extract. The amount of collagen in each extract was determined after hydrolysis at 105 °C for 16 hours in 6 mol/l HCl in sealed tubes from its hydroxyproline content multiplied by 7.46 (Neuman and Logan, 1950). Collagen extract was used for thiobarbituric acid test (TBA).

Preparation of collagen digests. — Insoluble collagen from skin specimens was prepared as described by Deyl and Adam (1976). Briefly, neutral salt-soluble and acid-soluble fractions were extracted and the remaining material was lyophilized. Collagen remaining after these extraction procedures was considered insoluble and represented over 80% of the original skin collagen. In aortal specimens, insoluble collagen was prepared by the method of Faris et al. (1978), in which elastase is used to remove the bulk of aortal elastin. The recovery of collagen by this method was better than 85%.

The insoluble fraction of skin or aorta collagen was washed twice with distilled water and centrifuged at 75,000 × g for 20 min at 4 °C. The pellet was suspended in 20 ml of 1.0 mol/l NaCl and sonicated until a fine suspension was obtained. Five hundred microliters were taken to determine the total amount of collagen present in the sample. A volume equivalent to 10 mg of collagen was then centrifuged at 20,000 × g for 20 min at 4 °C. The pellet was resuspended in 1.0 ml of 0.01 mol/l $CaCl_2$/0.02 mol/l Tris HCl pH 7.55 containing 0.05% toluene to prevent bacterial growth. To each sample 0.5 ml of a solution containing 1 mg purified collagenase (type CLS, Millipore, Worthington, PA) dissolved in the above specified Tris HCl buffer was added. The samples were shaken at 37 °C for 24 hours. An insoluble pellet accounting for less than 5% of total collagen was afterwards removed by centrifugation. Aliquots (50 μl) of the supernatant were used for the determination of the amount of digested collagen. The remaining supernatant served for spectroscopic and fluorescence measurement and for the determination of Amadori products.

TBA (2-thiobarbituric acid) test. — The 2-thiobarbituric acid test (Rosenberg et al., 1979) for determining the presence of 5-hydroxymethylfurfural (HMF) was performed by mixing 1 ml of the collagen extract (obtained by autoclaving) with 0.5 ml 0.3 mol/l oxalic acid and heating for 1 hour in a boiling water bath. After the samples were cooled to room temperature, 0.5 ml of 40% trichloroacetic acid was added and the resulting precipitate removed by filtration. After the addition of 0.5 ml of 0.05 mol/l TBA, the solution was incubated at 40 °C for 30 min. The absorbance at 443 nm then was measured and evaluated according to the standard curve.

Assay of the Amadori product. — Amadori products after borohydride reduction were assayed by affinity chromatography according to Brownlee et al. (1984) using the Glyco.gel Test Kit (Pierce, Rockford, IL). An amount equivalent to 200 μg of collagen (28 μg of hydroxyproline) was pipetted into a 10 ml test tube and brought to 0.5 ml by addition of buffer H from the kit. Then the borohydride reduction was performed with approximately 200 M excess borohydride over the total number of amino groups per collagen α-chain. To each tube was added 50 μl of 0.001 mol/l NaOH containing 256 μCi of (^{3}H)$NaBH_4$ (ICN, Covina, CA, specific activity 50 mCi/nmol). Reduction was carried out for 10 min at room temperature, 50 min at 4 °C, and brought to an end with 50 μl of 6 mol/l HCl after addition of 50 μl of n-pentanol. The solution was four times diluted with 1 ml of distilled water and evaporated with a Speed Vac concentrator (Savant, Hicksville, NY) to diminish the contents of volatile components. All steps described above were carried under a hood to prevent contamination of the lab with radioactivity. The dry residue was acid-hydrolyzed with 6 mol/l HCl for 12 hours at 110 °C, and afterwards the HCl was evaporated. The evaporation sequence was repeated five times to decrease the background radioactivity. The residue was dissolved in 0.5 ml of 0.25 mol/l ammonium acetate (Pierce buffer) and loaded onto a Glyco.gel minicolumn (Pierce). The column was washed with 20 ml of buffer and the Amadori products were eluted with 5 ml of 0.2 mol/l sorbitol. Three ml of the eluate were mixed with 17 ml of Hydrofluor (National Diagnostics, Sommerville, NY), and tritium activity was counted with a Beckman LS liquid scintillation counter. The calibration line was obtained with borohydride-reduced and acid-hydrolyzed samples of α-formyl-ε-fructosyllysine prepared in the laboratory. Nonspecific incorporation of tritium into collagen as estimated by the radioactivity not retained on the

column was 92.3–95.7% with the samples studied. The background activity was 34% of total counts incorporated into our samples (25% with the samples obtained from diabetic animals); although quite high, it was constant during the preliminary tests (CV = 9.6; N = 6).

Spectroscopical measurements. — Absorption at 350 nm was measured with a Zeiss PM6 spectrophotometer; fluorescence ($370_{ex}/440_{em}$ nm) was measured with the Perkin-Elmer fluorimeter model 204.

RESULTS

As indicated by the data shown in Table 1, a substantial age-dependent increase of glycation was found in skin and aorta collagen preparations from rats and mice (short-living species) between 0.25 and 2 years of age. In animals 5 years old and above (dog, cat, horse, and in human necroptic samples) no distinct age-dependent difference was detected. Two methods used to estimate the level of collagen glycation, namely the thiobarbituric acid test and the assay for hexosyllysine (Amadori conversion product) were in reasonable agreement, although the determination of the Amadori conversion product resulted in roughly 50% lower values as obtained with the thiobarbituric acid test. The amount of nonenzymatically bound glucose in skin collagen was about one half of the amount bound in the aortal preparations in all species and age groups investigated.

There is a reasonable amount of evidence in the literature that the glycated lysine residues in collagen undergo further conversion through the Amadori products to pigmented and/or fluorescent compounds. The chromophore in question was shown to exhibit a distinct absorbance at 350 nm and fluorescence at 370/440 nm. When our collagen preparations were evaluated on the basis of spectral measurements, a distinct increase with age (namely on mice and rats) in both the absorbance at 350 nm and fluorescence at 370/440 nm was observed accordingly.

DISCUSSION

As shown by our data, there is a build-up of the collagen-bound extracellular chromophore with the age of the animals that presumably arises from the Maillard reaction. The differences seen in collagen preparations between different animal species of comparable age and for similar expected life span are minimal. This may be explained either by assuming that the turnover of collagen or the rates of advanced glycosylation product removal are similar in the different species investigated. The differences in collagen glycation in different tissues are, however, quite noticeable, as demonstrated with aortae and skin preparations.

The results of the TBA test can be affected by the presence of products arising from the free radical peroxidation by lipids. It was reported in Kikugawa and Beppu (1987) that when alkanals and alkenals react with TBA, they produce

Table 1. Parameters of Glycation in Aortal and Skin Collagen Preparations

Animal[a]	Age (year)	nmol HMF (mg kollagen)$^{-1}$	nmol Hexosyllysine (mg kollagen)$^{-1}$	Absorbance[b] 350 nm	Relative Fluorescence $370_{ex}/440_{em}$	n
Mouse	0,25	11,98 ± 2,20	5,87 ± 0,76	0,070	35 ± 12	10
		4,98 ± 0,75	2,34 ± 1,01	0,100	45 ± 15	
Rat	0,25	12,25 ± 2,15	5,73 ± 0,89	0,062	34 ± 13	10
		5,54 ± 1,19	2,20 ± 0,92	0,112	42 ± 13	
Mouse	2	19,87 ± 3,12	9,23 ± 1,44	0,192	120 ± 12	10
		11,86 ± 2,79	6,59 ± 1,01	0,227	100 ± 11	
Rat	2	20,37 ± 3,15	9,08 ± 1,33	0,189	113 ± 11	10
		13,52 ± 2,03	6,59 ± 1,12	0,236	124 ± 17	
Miniature swine	2	21,15 ± 2,75	8,76 ± 1,12	0,180	125 ± 13	3
		12,11 ± 2,04	5,78 ± 1,02	0,230	135 ± 15	
Cat	7	22,93 ± 2,15	11,64 ± 1,09	0,195	132 ± 17	5
		13,26 ± 2,21	7,28 ± 1,39	0,235	163 ± 15	
Dog	5	24,12 ± 3,15	9,57 ± 1,22	0,187	124 ± 11	3
		12,89 ± 2,21	6,78 ± 1,21	0,231	145 ± 17	
Dog	7	25,12 ± 2,87	9,55 ± 2,03	0,187	126 ± 14	5
		12,96 ± 1,98	6,82 ± 1,33	0,230	155 ± 17	
Horse	11	26,72 ± 3,12	13,26 ± 2,15	0,200	135 ± 18	3
		14,03 ± 2,11	7,56 ± 2,02	0,255	187 ± 20	
Horse	18	23,18	14,17	0,202	144	1
		14,42	7,89	0,257	195	
Human	20–22	22,82 ± 2,17	15,22 ± 1,29	0,204	142 ± 16	3
		14,58 ± 3,11	7,98 ± 2,06	0,255	190 ± 21	
Human	73–75	29,13 ± 3,15	15,73 ± 1,22	0,229	178 ± 18	3
		15,06 ± 4,11	7,94 ± 2,12	0,290	248 ± 25	

Note. The data represent average *SD* from n estimates (see last column).

[a] For each group, distinct values are given for aortal (first line) and skin (second line) collagen.

[b] Absorbance values represent the average from two independent preparations.

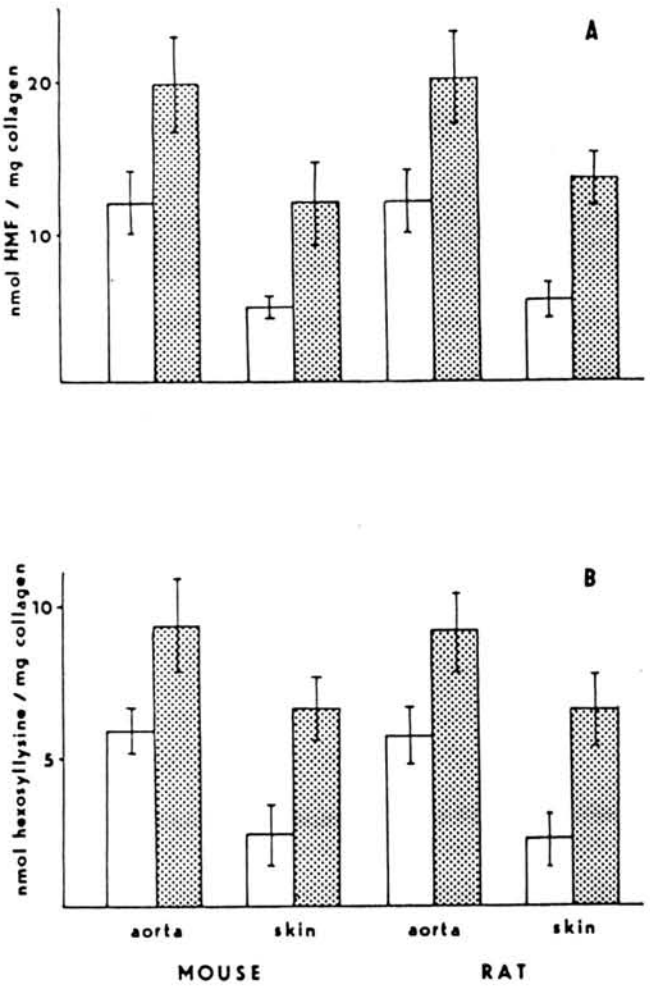

Figure 1. Effect of age on collagen glycation determined by TBA test (panel A) and affinity chromatography (panel B). Open columns: young animals (0.25 year); Dotted columns: old animals (2 years). All differences between young and old animals are significant ($p < .005$).

yellow to red colored pigments. On one hand, it has to be taken into consideration that our preparations were defatted prior to collagen isolation. Thus such TBA reactivity could arise only from protein-bound lipid peroxidation products which withstand the preparation procedure. Such a situation can occur, and in this case the results obtained by the TBA test would be higher than values obtained by direct chromatographical estimation of the Amadori rearrangement product (ε-hexosyllysine). Such differences were observed in our preparations. On the other hand, consistent differences of this type may also arise from the methods used. Kumar et al. (1988) reported that the values obtained by the TBA test and fructosamine assay differ about eight times if the glycated samples are evaluated by the fructosamine method.

Cannon and Davison (1976) have shown that collagen carbonyl groups do react with Tris HCl. As Tris HCl is used as a buffering medium for collagen digestion with collagenase, we have performed a control experiment in which HEPES was used instead. As the absorbance level measured at 350 nm after digestion was only 10% lower with HEPES preparation, we think that in our case the results are not influenced significantly by using either buffering agent.

In the case of diabetes, augmented collagen glycation reflects an increased level of blood glucose and can be correlated with an increased level of glycated hemoglobins; in our animals the blood glucose level does not show detectable alterations during the life span, and the occurrence of glycation products reflects probably the exposure time. As specified by Oimomi et al. (1986, p. 697), "the increase of glycation in tissues appeared to be due to aging, not due to the increased glucose level." In this context it would be more precise to say that the increased glycation, at least of collagen, is the result of its slow metabolic turnover (as compared to, e.g., hemoglobin), which may be further slowed down by cross-links formed from glycated lysines in the subsequent steps of the Maillard reaction (Waller et al., 1983). Indeed, as demonstrated by Miyahara et al. (1982), skin collagen from aged individuals is incomparably more resistant to a proteolytic attack as compared to preparations obtained from young individuals. The determination particularly of the UV absorbing and fluorescent products formed in the last stage of the Maillard reaction appears important in evaluating the aging process as these compounds may be considered as typical age pigments, although located in the extracellular space. However, no clear-cut biochemical evidence that the chromophore is the final result of glycation reactions is currently available.

In more rapidly metabolized proteins the results of age-dependent glycation, as with hemoglobin, for instance, are controversial. While Oimomi et al. (1986) were unable to demonstrate any increase in the proportion of glycated hemoglobins with age, Arnetz et al. (1982) were able to show an increase in HbA_{1c} in older nondiabetic people (see also Bunn, 1981; Graf et al., 1978; Muller et al., 1981). It is currently agreed that the proportion of HbA_{1c} represents a marker of blood glucose level in the preceding 8–12 weeks (Arnetz et al., 1982). The situation with collagen may be well the same, i.e., differences in the proportion of glycated collagen in older individuals may reflect a higher integrated value of blood glucose in older nondiabetic individuals; on the contrary, with hemoglobin this is likely to reflect a longer preceding period due to a slower metabolic turnover of collagen.

The data presented in Table 1 also show a distinct age-dependent increase in the absorbance at 350 nm and in the $370_{ex}/440_{em}$ nm fluorescence both in aortae and skin of rats and mice between 0.25 and 2 years of age, whereas results obtained with the TBA test or by determination of ε-hexosyllysine show that the levels of absorbance and fluorescence were always higher for skin than for aortae preparations. In samples obtained from animals or human beings older than 5 years the increase in absorbance and fluorescence level is small; the only exception was found for 5- and 7-year-old dogs, where no significant differences were observed; perhaps the age difference of only two years is too small to be reflected in the investigated parameters.

Based on the assumption of constant blood glucose level, the extent of glycation is then solely dependent on the exposure of nonglycated collagenous structures to blood glucose. In parallel, collagen structures are polymerized

through aldimine-type cross-links. The turnover of such polymerized collagen is then considerably slowed down, finally exhibiting a half-time of roughly two and a half years (Pelkonen and Kivirikko, 1970). Thus there is enough time available for the glycation to proceed on this slowly metabolized protein. Also, the rather rapid increase of bound hexose in early life phases is in agreement with rapid increase of the amount of Amadori product. Next it is feasible to speculate that the reaction sequence continues so that the investigated chromophore (fluorophore) is formed. If one assumes that the latter step is related to cross-linking, then it would be acceptable to also assume a further slowdown of metabolic turnover for such modified species. Thus the protein is preserved in the tissue for a longer period of time and consequently glycated to a higher degree as revealed by our data. The conversion of bound hexose to the chromophore (fluorophore) must proceed with a slower rate than glycation, as otherwise a constant (or even zero) level of bound hexoses would be revealed.

The differences between skin and aortic collagen glycation may reflect either differences in collagen turnover in these two tissues, or may reflect the fact that the macrophage removal process (Vlassara et al., 1985) is more efficient in skin. However, this regards only the catabolic processes. The observed difference may also reflect the fact that, in aorta, higher cumulative amounts of glucose over a definite period of time are typically available to promote tissue collagen glycation.

CONCLUSIONS

Based on the data from this investigation, the following conclusions can be made:

1. Glycation and browning are comparable in collagen specimens from different species of comparable age.
2. Glycation and browning increase markedly in the first 5–10 years.
3. The extent of glycation of aortal collagen is about twice that of skin collagen.
4. Browning as followed by fluorescence is comparable in aortal and skin collagen (except below 10 years). In contrast, browning as measured by the absorbance at 350 nm is lower in aortal than in skin collagen.

These findings seem to indicate that at any level of glycation there is comparably more absorbance and fluorescence in skin than in aortal collagen. The lower extent of glycation of skin collagen, therefore, appears to be caused by removal of glycated sites by browning. (At glycation levels up to 10 nmol hexosyllysine/mg collagen, the fluorophore content of skin collagen is approximately twice that of aortal collagen.) The lack of an increase in glycation or browning in older collagen indicates that by this time glycation, browning, and turnover have reached a steady state. These results are in agreement with data published on human lens proteins (Patrick et al., 1990).

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