Age-related Changes in Collagen : The Identification of Reducible Lysine-Carbohydrate Condensation Products

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Received May 12, 1972

In recent publications (Bailey et al 1969; Bailey & Shimokomaki, 1971) we have reported studies of the age-related changes in the reducible cross-links of several types of collagens. It was shown that with all the tissues studied there was a decrease with age in the amounts of all these intermolecular cross-links, presumably by conversion of the aldimine bond to a more stable non-reducible form. Since these changes inversely paralleled the rate of growth, the process was considered an essential step in the maturation of collagen.

In addition, it was found that two of the reducible components, designated Fraction A, increased with age. This phenomenon was apparent not only in the different tissues examined, but also in different species of animal and in man. Thus, an increase in Fr. A appears to be a general characteristic of ageing and it was for this reason that further studies of the nature of these components have been carried out. This paper briefly describes their isolation and identification from mature bovine skin.

Experimental

Sixteen year old bovine skin (approx. 2 Kg wet wt.) was prepared and reduced with potassium borotritide by the methods previously described (Bailey et al 1970). After hydrolysis of the reduced tissue in boiling 6 N HCl for 24 hr. the evaporated hydrolysate was submitted

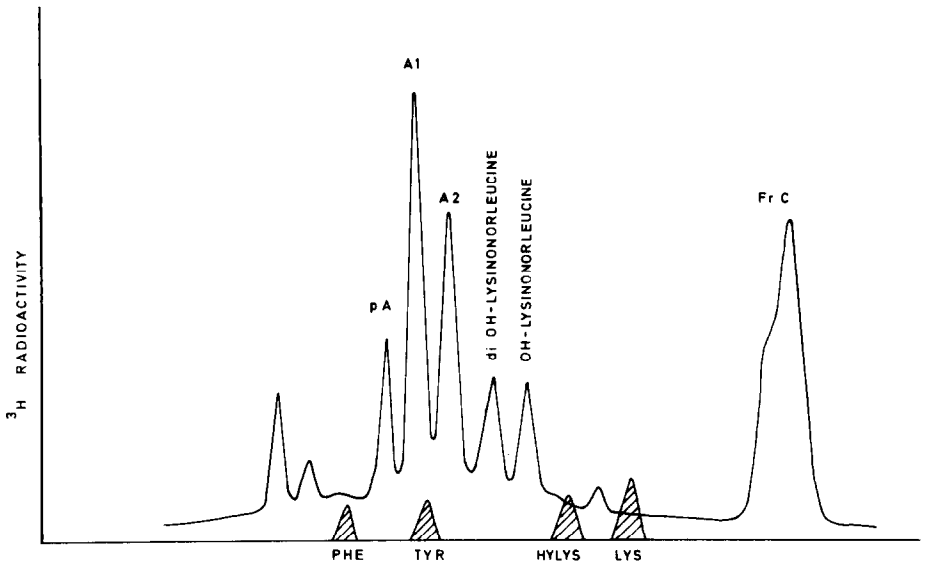

Figure 1. The elution pattern with volatile buffers of the ^{3}H-labelled components of an acid hydrolysate of reduced 16 yr. bovine skin relative to that of normal amino acids (hatched areas).

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to displacement chromatography on cation-exchange resin using the procedures previously described (Bailey et al 1970). The band from this chromatogram containing the radioactive components comprising Fr. A were chromatographed on a column (16 cm^2 x 90 cm) of Sephadex G 10 in 5% acetic acid and the higher molecular weight fraction then submitted to ion-exchange elution chromatography using pyridine-formate buffers. Further fractionation of the components was achieved using an extended basic column of a Locarte amino acid analyser. Final purification of the desalted components was effected by chromatography with a column (2 cm^2 x 90 cm) of Sephadex G 10 in 0.5% acetic acid.

Results

Characterization of components in reduced collagen. Fig. 1 shows the elution pattern with volatile buffers of the acid hydrolysate of the

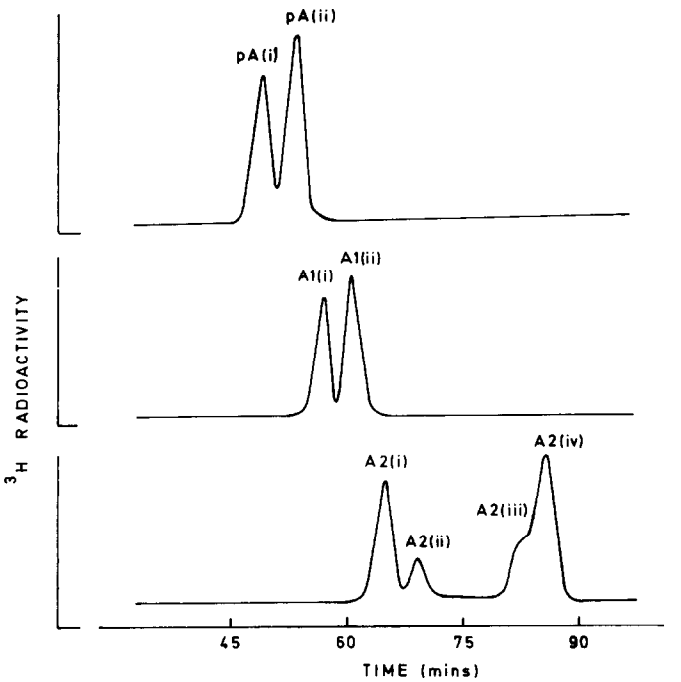

Figure 2. The relative chromatographic behaviour of fractions pA, A1 and A2 using an extended basic column of a Locarte amino acid analyser.

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reduced bovine skin. In addition to the main components of Fr. A, designated A1 and A2, a slightly more acidic component (pA) was present, the proportion of which was found to be dependant on the precise conditions of acid hydrolysis.

In Fig. 2 are shown the elution patterns of the components of Fr. A using the basic column of the Locarte analyser. Both fractions pA and A1 separated into two components whereas fraction A2 consisted of four main components.

On treatment of the radioactive fractions A1(i) and A1(ii) with periodate (0.05 M; pH 5.25; 10 mins; room temp.) both of these compounds were completely degraded with the production of non-radioactive lysine. These results demonstrated that the compounds must be condensation products involving the ϵ-NH_2 group of lysine since, if lysine-aldehyde (δ-semi-

aldehyde of α amino adipic acid) had been involved the lysine produced by periodate treatment would have been radioactive.

Analysis by mass spectrometry of the trifluoroacetylated methyl esters of components A1(i) and A1(ii) showed that both of these derivatives produced mass ions at m/e = 996. This, together with their fragmentation patterns, was consistent with the compounds being derivatives of Nε-1-(1-deoxyhexitol)-lysine. Trifluoroacetylated methyl ester derivatives of fractions A2(i), A2(iii) and A2(iv) all gave spectra with mass ions at m/e = 786. The spectra were consistent with these compounds being mono-anhydro-derivatives of Nε-1-(1-deoxyhexitol)-lysine.

Chemical synthesis. In order to determine which of the hexitols were involved, syntheses were carried out of the hexitol-lysine derivatives of mannose, glucose and galactose, these three being the major hexose constituents of collagen (Grant & Jackson 1968; Spiro, 1969). Solutions containing hexose and lysine in a 2:1 molar ratio were incubated at room temperature for 4 hrs at pH 7.9 and then reduced with potassium borotritide. The products were isolated by the methods already described. The fact that identical compounds were obtained by condensing the hexoses with polylysine (Yeda, Israel) showed that substitution at the ε-amino group rather than the α-amino group had occurred.

Fig. 3 shows the chromatographic elution patterns of the synthetic hexitol-lysines relative to those of fractions A1 and A2 together with the elution patterns of acid hydrolysates (boiling 6 N HCl; 12 hrs) of the synthetic materials. Nε-mannitol-lysine was the only derivative which co-chromatographed with fraction A1(i) whereas both Nε-glucitol-lysine and Nε-galactitol-lysine co-chromatographed with fraction A1(ii). Derivatives of the synthetic materials gave identical mass spectra to those obtained for the collagen-derived compounds. The anhydrides produced by hot acid treatment (Foster & Overend, 1951) of the synthetic hexitol-lysines accounted for peaks (i), (iii) and (iv) of fraction A2

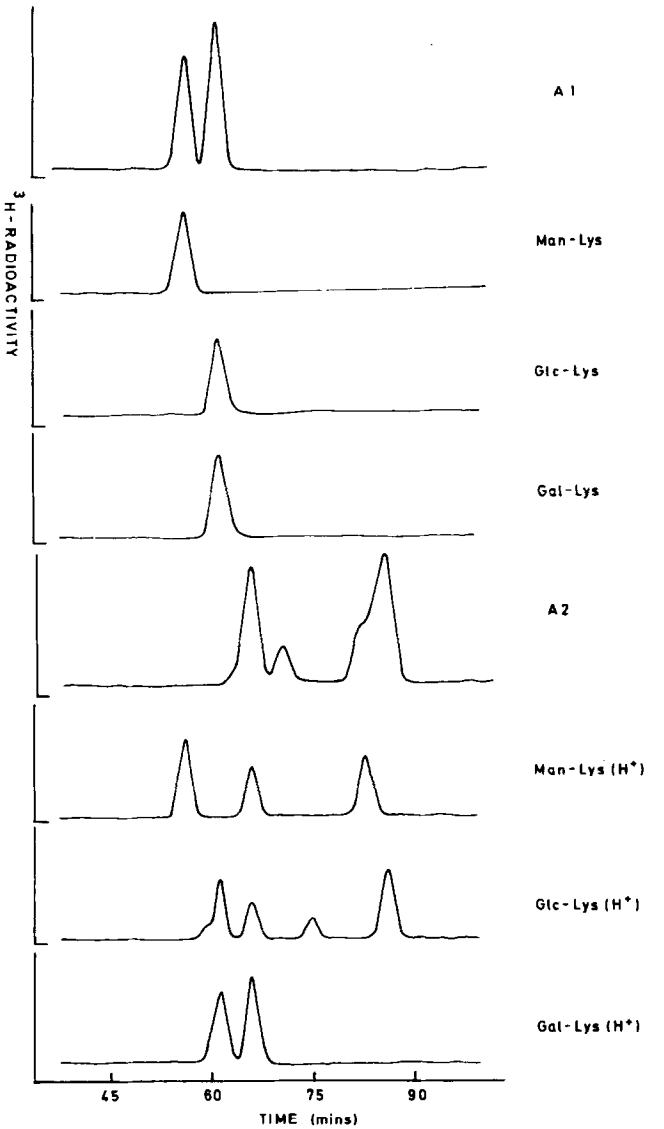


Figure 3. The elution patterns using the Locarte Analyser of fractions A1 and A2 in comparison with those of the synthetic hexitol-lysine derivatives from mannose (Man-lys), glucose (Glc-lys), and galactose (Gal-lys), and with those of acid hydrolysates of the synthetic materials (Man-lys H^+, Glc-lys H^+ and Gal-lys H^+).

(Fig. 3) and the identity of the synthetic and natural anhydrides was confirmed by mass spectrometry.

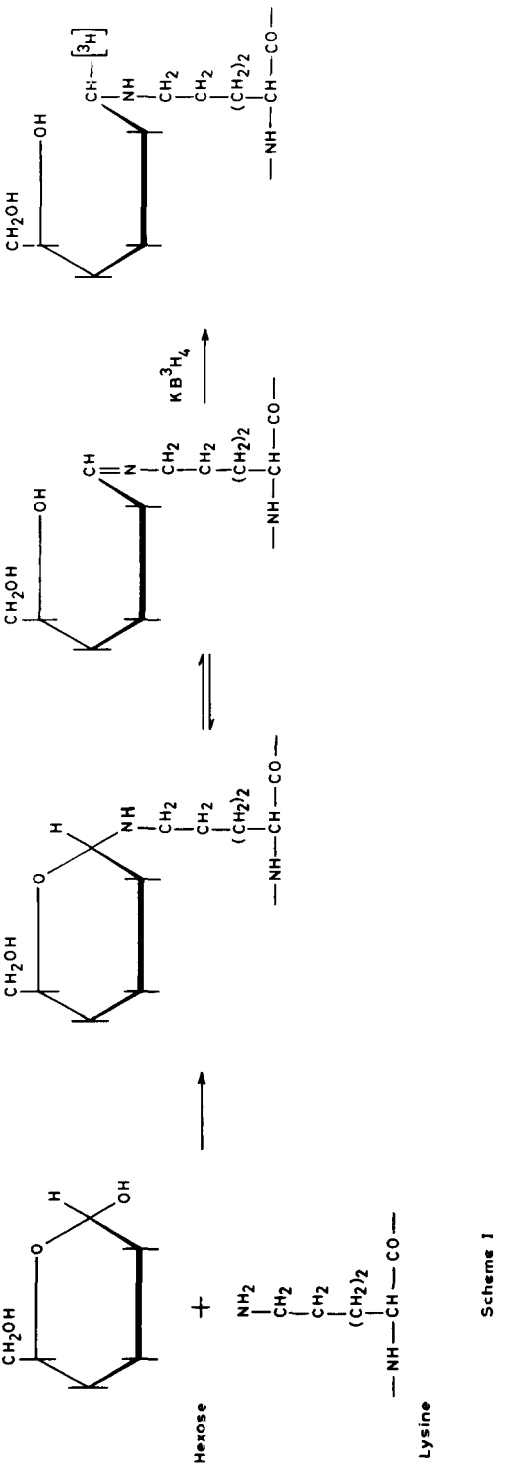
Since both glucitol- and galactitol-lysines co-chromatographed with fraction A1(ii) under the conditions used and since each produced an anhydride which co-chromatographed with fraction A2(i), it was necessary to determine whether both or only one of these derivatives was present in collagen. Comparison of the periodate sensitivities under standard conditions showed that the anhydride of galactitol-lysine was completely destroyed whereas the chromatographically identical anhydride from glucitol-lysine appeared to be virtually unchanged by periodate treatment. Since fraction A2(i) from skin collagen was found to be stable to periodate it may be concluded that the component A1(ii) must be predominantly Nε-glucitol-lysine.

Analysis by mass spectrometry together with periodate studies have established that the components pA(i) and pA(ii) are the corresponding hexitol derivatives of hydroxylysine. Furthermore, synthetic Nε-mannitol-hydroxylysine and Nε-glucitol-hydroxylysine have identical properties to those of fractions pA(i) and pA(ii) respectively.

Discussion

It has been shown that the radioactive component designated fraction A1 of borotritide-reduced bovine skin is composed of Nε-mannitol-lysine and Nε-glucitol-lysine (Scheme 1). The anhydro-derivatives which comprise fraction A2 are presumably solely artefacts of the acid hydrolysis procedure. As might be expected, analogous derivatives involving hydroxylysine are also present but in considerably smaller proportions.

Since these compounds were isolated from reduced tissue, they must have been present initially as Nε-lysine-glycosylamines, probably in equilibrium with their Schiff base forms. That such glycosylamines do exist under physiological condition has been demonstrated for normal



Scheme 1

human haemoglobin (Bookchin and Gallop, 1968) where some 6% of the molecules were found to have hexose attached to the N-terminal valyl residue of one of the β chains. The non-reduced glycosylaminated haemoglobin was sufficiently stable to allow chromatographic and electrophoretic separation at neutral or slightly alkaline pH and to allow detection of the blocked N-terminal peptide by peptide mapping after tryptic hydrolysis.

At the present time it is uncertain as to whether these compounds exist naturally in collagen or whether they are produced during the preparation of the tissue. Although glucose and galactose have been shown to be O-glycosidically linked to hydroxylysine (Spiro, 1969) the possible attachment to collagen of the remainder of these hexoses, including mannose has not yet been clarified and may well involve the glycosylamine linkage demonstrated here.

The possible physiological significance of these results for collagen cannot as yet be assessed. It seems certain, however, that the isolated compounds do not constitute crosslinks between collagen molecules. The observed increase with age in the amounts of these glycosylamines might indicate a systematic increase in the binding of collagen to polysaccharides, a fact which could have considerable influence on the organisation of the fibrils. Further studies are being carried out to elucidate the sites of attachment and nature of the carbohydrate appendages.

Acknowledgements

We thank Professor T. K. Ewer and Dr. S. M. Partridge FRS for their generous encouragement and the Arthritis and Rheumatism Council for financial support.

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