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Biochemical and morphological modifications in rabbit Achilles tendon during maturation and ageing

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1. Achilles tendons of foetal, newborn, adult and old rabbits were examined by electron microscopy after staining by conventional methods or with the periodate/silver/methenamine technique. 2. The mean diameter of collagen fibrils increased with age whereas silver/methenamine-positivity became less evident. 3. Biochemical analyses showed a great decrease of the concentration of glycoproteins and galactosamine-containing glycosaminoglycans. 4. Collagen content increased with maturation and ageing of the tissue. 5. The extent of glycosylation of collagen hydroxylysine residues was also age-dependent; the total amount of hydroxylysyl glycosides rapidly decreased in the last days of prenatal life and in the first months after birth, corresponding to the rapid growth in collagen fibre diameter. 6. The hydroxylysyl diglycoside concentration decreased more markedly than that of the monoglycoside, thus indicating a possible gradual removal of the monosaccharide units. A role for the extent of glycosylation of tropocollagen molecules in fibre organization was suggested.

In recent years some structural and morphological aspects of adult bovine Achilles and rat tail tendon were carefully studied by several authors and, in particular, the different non-collagenous components of the matrix and their relationships with collagen fibrils were investigated.

A low-molecular-weight proteodermatan sulphate and a high-molecular-weight proteochondroitin sulphate were isolated and characterized from adult bovine tendon (Anderson, 1975; Anderson & Labedz, 1979; Anderson & Khullar, 1980). A soluble glycoprotein closely associated with the proteodermatan sulphate fraction was also extracted and purified (Anderson, 1975). This molecule appeared to be able to affect *in vitro* the formation of collagen fibrils by decreasing their growing rate (Anderson *et al.*, 1977).

A similar effect can be exerted by glycosaminoglycans; in fact, Keech (1961), while studying the appearance under the electron microscope of collagen fibrils regenerated from solution, observed that the presence of chondroitin sulphate in the precipitating medium greatly increased the rate of fibril formation and lowered their diameter by a factor of 3. Wood (1960, 1964) showed that the rate of reconstitution of collagen fibrils and hence their diameter was related to the chemical structure of polyanions present: chondroitin 4- and 6-sulphates initiated a rapid fibril formation, whereas hyaluronic acid, dermatan sulphate and keratan sulphate did not affect the precipitation rate. It was suggested, therefore, that the presence of high concentrations of chondroitin sulphate in a tissue could result in fine-fibril formation, whereas hyaluronic acid, dermatan sulphate and heparan sulphate may permit the lateral alignment of tropocollagen molecules to give broader fibrils.

Moreover, Öbrink (1973), in a study of the interactions *in vitro* between monomeric tropocollagen and glycosaminoglycans, demonstrated that, in the earliest stage of fibrillogenesis, the nature of the glycosaminoglycan present can affect fibre maturation and growth, and in these experiments iduronic acid-containing glycosaminoglycans showed a stronger interaction with collagen than did glucuronic acid-containing molecules.

These results obtained *in vitro* suggested the possibility of different collagen–proteoglycan inter-

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actions occurring with maturation and ageing of the tissue, when modifications of morphology and chemical composition of intercellular matrix occur.

In fact the regular pattern of proteoglycans associated with the surface of collagen fibres in rat tail tendon, described by Scott (1980) and Scott & Orford (1981), was demonstrated to change throughout life (Scott *et al.*, 1981*a*,*b*); they reported, in fact, an increase of mean collagen fibril diameter and a modification of proteoglycan content with age. Dermatan sulphate was the main glycosaminoglycan in the mature tendon, whereas chondroitin sulphate and hyaluronic acid were preponderant in foetal tissue.

Since a decrease in the ratio of proteoglycans to collagen and a relative decrease of chondroitin sulphate content were demonstrated (Scott *et al.*, 1981*a*,*b*), an inhibiting action of chondroitin sulphate-rich proteoglycan on fibril diameter growth was suggested.

Both qualitative and quantitative differences in glycoprotein and glycosaminoglycan content of tendon may, therefore, influence the nature of the collagen network laid down and play a different role during maturation and ageing of the tissue.

At present, however, it is not known if tendon collagen undergoes chemical modifications corresponding to the well-demonstrated increase of fibril diameter.

Since an inverse correlation between the amounts of neutral sugars associated with the collagen and the diameter of the fibrils was proposed (Morgan *et al.*, 1970), the glycoprotein component of the matrix and, in particular, the hydroxylysyl glycosides of collagen, appeared to represent an interesting subject of research.

Therefore, in the present paper, rabbit Achilles tendon was examined in view of the marked morphological changes observed in the cells and in matrix of this tissue during maturation, together with a decrease in periodate/Schiff-positivity, which suggested a possible decrease of the content of neutral-sugar-containing molecules (Puddu & Ippolito, 1974; Ippolito *et al.*, 1980). In order to localize and evaluate these compounds more specifically, morphological studies were carried out by using a periodate/silver/methenamine technique for electron microscopy (De Martino & Zamboni, 1967).

Biochemical investigations on glycosaminoglycan and glycoprotein components were also undertaken in an attempt to correlate all the biochemical changes of this tissue with the morphological ones and, since at present little is known about the behaviour of collagen glycosylation with ageing, collagen composition was studied in detail. In particular the hydroxylysyl glycosides were carefully examined in order to assess the possible role of these residues in growing collagen fibres and in matrix organization.

Materials and methods

Achilles tendons were obtained from foetal (23 and 27 days *post coitus*), newborn, young (2 months old) and old (4 years old) New Zealand White rabbits. The material from 23-days-foetal up to newborn rabbits was dissected out under a low-power dissecting microscope.

Ultrastructural methods

The tendons were prefixed *in situ* with the animal under general anaesthesia. The fixative was dripped on a small area of the tendon surface with a Pasteur pipette. The portion of tendon partially fixed was removed after 20 min of dripping and cut into small rectangular specimens. The fixation was then completed in a test tube. The following fixatives were used: (*a*) 4% (w/v) paraformaldehyde in Millonig (1961) buffer, pH 7.4 (fixation time 2 h at 4°C); (*b*) 2% (w/v) osmic acid in Millonig buffer, pH 7.4 (fixation time 1 h at 4°C).

After fixation the samples were thoroughly washed with Millonig buffer, pH 7.4 (10 min, three times), then with distilled water (10 min, three times) and afterwards dehydrated in ethanol [50, 70, 80 and 95% (v/v), 10 min each, followed by three passages of 15 min in 100% ethanol], cleared in toluene and embedded in Epon 812 in gelatine capsules.

Thin sections were partially stained with uranyl acetate and lead hydroxide. For the silver/methenamine reaction, 80 nm-thick sections of the paraformaldehyde-fixed tissue were picked up with copper wire and transferred to a Petri dish containing 2% (w/v) periodic acid. The sections were oxidized for 15 min at room temperature, thoroughly washed with distilled water, and then stained with a solution obtained by mixing 0.8 ml of 5% (w/v) sodium tetraborate decahydrate (Merck) in twice-distilled water with 10 ml of 5% (w/v) silver nitrate (Merck) in 3% (w/v) hexamethylenetetra-amine (Eastman Organic Chemicals) in twice-distilled water and diluting the final mixture with an equal volume of twice-distilled water. After incubation at 55°C for about 30 min the sections were rapidly rinsed in three changes of twice-distilled water and mounted on Formvar-coated copper grids. The sections were observed and photographed with a Siemens Elmiskop 101 electron microscope.

The diameters of collagen fibrils were measured on the electron micrographs of cross-sectioned tendons after calibration of magnification, and the average diameters were evaluated by measuring about 5000 fibrils in each age-group sample.

Biochemical methods

Insoluble collagen was obtained by the method of Sykes *et al.* (1976), modified as previously described (Cetta *et al.*, 1979). The tendons were dehydrated with chloroform/methanol (1:1, v/v), dried by evaporating the solvents and extracted for the soluble collagen in 0.15 M-NaCl in 50 mM-Tris/HCl buffer, pH 7.5. The insoluble material was digested with pepsin (dry tissue/enzyme, 10:1, w/w), then the solubilized collagen was purified by repeated cycles of precipitation in 2 M-NaCl and redissolution in 0.5 M-acetic acid. The extracted collagen appeared to be homogeneous on electrophoresis (Furthmayr & Timpl, 1971).

In order to study glycosaminoglycans and glycoproteins, the tendons were treated overnight with a mixture of methanol/chloroform (1:1, v/v) by using 300 vol. of this solution/g of wet tissue with stirring at 4°C, then dried by evaporating the solvents under vacuum. Afterwards the tissues were suspended in 0.1 M-sodium acetate (pH 5.6)/5 mM-EDTA (tetrasodium salt)/5 mM-cysteine hydrochloride and digested with papain as described by Scott (1960).

After enzyme denaturation in boiling water for 2 min, the digested material was centrifuged at 30000 *g* for 1 h, at 4°C and the precipitate discarded. From the supernatant, containing glycosaminoglycans and digested glycoproteins, the glycosaminoglycans were precipitated by adding 10% (w/v) cetylpyridinium chloride to obtain a final concentration of about 0.5%. After the mixture had been left overnight at 4°C the precipitate was separated by centrifugation and washed twice with 10% (w/v) potassium acetate in 95% (v/v) ethanol, twice with 95% ethanol and finally dried with diethyl ether. The supernatant, containing digested glycoproteins, was then analysed separately.

Analytical determinations

Hexosamines were determined on a Technicon NC-2 liquid chromatograph after hydrolysis in 4 M-HCl for 7 h at 105°C; uronic acids were assayed as described by Bitter & Muir (1962). Hydroxylysyl glycosides were determined in extracted collagen or in dry tendon by a modified method of Isemura (1976). Samples containing 25–200 nmol of glycosides were hydrolysed at 110°C for 24 h, with 0.5–2 ml of 2.5 M-NaOH in polypropylene tubes sealed inside Pyrex tubes, under N_2. After hydrolysis the alkaline solution was diluted with water (about 1:8), acidified with 5 M-acetic acid and adjusted with 6 M-HCl to pH 2–2.5. This solution, prepared just before the analysis, was applied to a column (1 cm × 5 cm) of AG or Dowex 50 (X 8; 200–400 mesh; H^+ form) resin. The resin was washed sequentially with 50 ml of water and 30 ml of 8% (v/v) freshly distilled pyridine to eliminate neutral and acidic amino acids. The hydroxylysyl glycosides were then eluted with 30 ml of 3 M-NH_3.

The NH_3 fraction was dried and, after addition of 1 ml of 0.2 M-sodium citrate buffer, pH 2.2, an appropriate portion was analysed with sodium citrate buffer, pH 5.28, as eluent under the same experimental conditions normally used for hexosamines. The colorimetric response per mol of both diglycoside and monoglycoside was considered the same as that of hydroxylysine. Hydroxyproline was assayed after acid hydrolysis of the specimen in 6 M-HCl as described by Huszar *et al.* (1980). The collagen content was extrapolated by multiplying the hydroxyproline content by the factor 7.40. This value was calculated on the basis of a mean hydroxyproline content (98 residues/1000 residues) in collagen type I.

Results

The Achilles tendon of newborn rabbits showed collagen fibrils of fairly uniform size that in cross-section appeared to be separated by electron-lucent spaces containing finely granular material. Fibril diameter ranged from 18 to 55 nm, and 37 nm-thick fibrils accounted for the 70% of the whole population. The fibrils were found to be strongly and rather uniformly silver/methenamine-positive, whereas no positive material was found in the interfibrillar matrix (Plates 1*a* and 1*b*). In foetal tendons no obvious differences in collagen-fibre diameter appeared, though a sharper positivity of the silver/methenamine reaction in comparison with tendons of newborn animals seemed to be present.

EXPLANATION OF PLATE 1

Rabbit tendon matrix in cross section

(*a*, *b*) Newborn tendon. The collagen fibrils have a fairly uniform diameter ranging from 18 to 55 nm (*a*) and they are strongly and rather uniformly silver-positive (*b*). No silver-positive material is present in the interfibrillar spaces. (*c*, *d*) Young tendon. The collagen fibrils vary in diameter, ranging from 18 to 166 nm (*c*). The silver-positivity is sharply decreased in the thicker fibrils, whereas the thinner ones keep their strong positivity (*d*). The silver grains are mainly located at the periphery of the fibrils. (*e*, *f*) Old tendon. The collagen fibrils further increase in diameter, ranging from 18 to 203 nm (*e*). The silver-positivity is slightly decreased in comparison with the young tissue. (*a*, *c*, *e*: fixed and stained with 2% osmic acid, uranyl acetate and lead hydroxide; *b*, *d*, *f*: fixed and stained with 4% paraformaldehyde and periodic acid/silver/methenamine). Magnification: *a*, *b*, *c*, *e*, about ×68000; *d*, *f*, about ×136000).

Collagen fibres varied very much in diameter and were more closely packed in young animals. The size ranged from 18 to 166nm, and the two more frequent populations of fibres in this group measured 55nm (16%) and 97nm (16%). Staining with silver/methenamine was much less evident at this age (2 months), as the electron-dense grains in cross-sections of collagen fibrils appeared to be markedly decreased (Plates 1*c* and 1*d*). The old tendon showed collagen fibrils ranging from 18 to 203 nm, and a size of 37 nm was the most frequently observed (19.5%). A further decrease of the silver-grain density in comparison with young tissue was observed (Plates 1*e* and 1*f*).

In both young and old tissues the silver/methenamine-positivity of collagen fibrils gradually became less evident from thin (37 ± 18 nm) to middle (74 ± 20 nm) and thick (148 ± 18 nm) fibres. The silver/methenamine-positivity of both middle and thick fibrils appeared to decrease from the centre towards the periphery, where a coat of silver grains was always present.

Biochemical analyses showed that the hexosamine content, as determined on the papain digest of whole tissue, rapidly decreased until 2 months after birth and then slightly afterwards (Fig. 1*a*). A significant variation of the ratio glucosamine/galactosamine from young to old animals was also observed (Fig. 1*b*). This result may be due to the different behaviour of the glycoprotein fraction in comparison with glycosaminoglycans; in fact the 'glycosaminoglycan fraction' (cetylpyridinium chloride-precipitable material), which showed a strong decrease of total hexosamine content (Fig. 1*c*), also showed a greater decrease in galactosamine, which may explain the observed increase in the glucosamine/galactosamine ratio (Fig. 1*d*).

In agreement with the data of Scott *et al.* (1981*a*,*b*), the uronic acid content markedly diminished during the first 2 months of postnatal life and slightly afterwards, confirming the decrease of glycosaminoglycan content of the tendon indicated by hexosamine determination in this fraction.

The modification of the ratio glucosamine/galactosamine and the consideration of the fact that chemical analysis of cetylpyridinium chloride-precipitated glycosaminoglycans showed constant equimolecular amounts of hexosamines and uronic acids suggested that the decrease in glycosaminoglycan content of tendon during the life span especially involved galactosamine-containing molecules (chondroitin sulphate and dermatan sulphate).

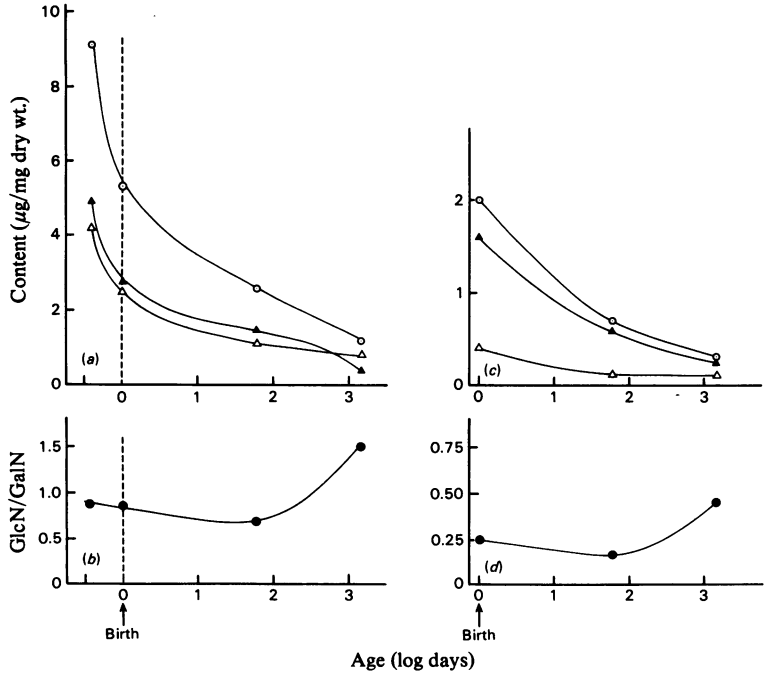
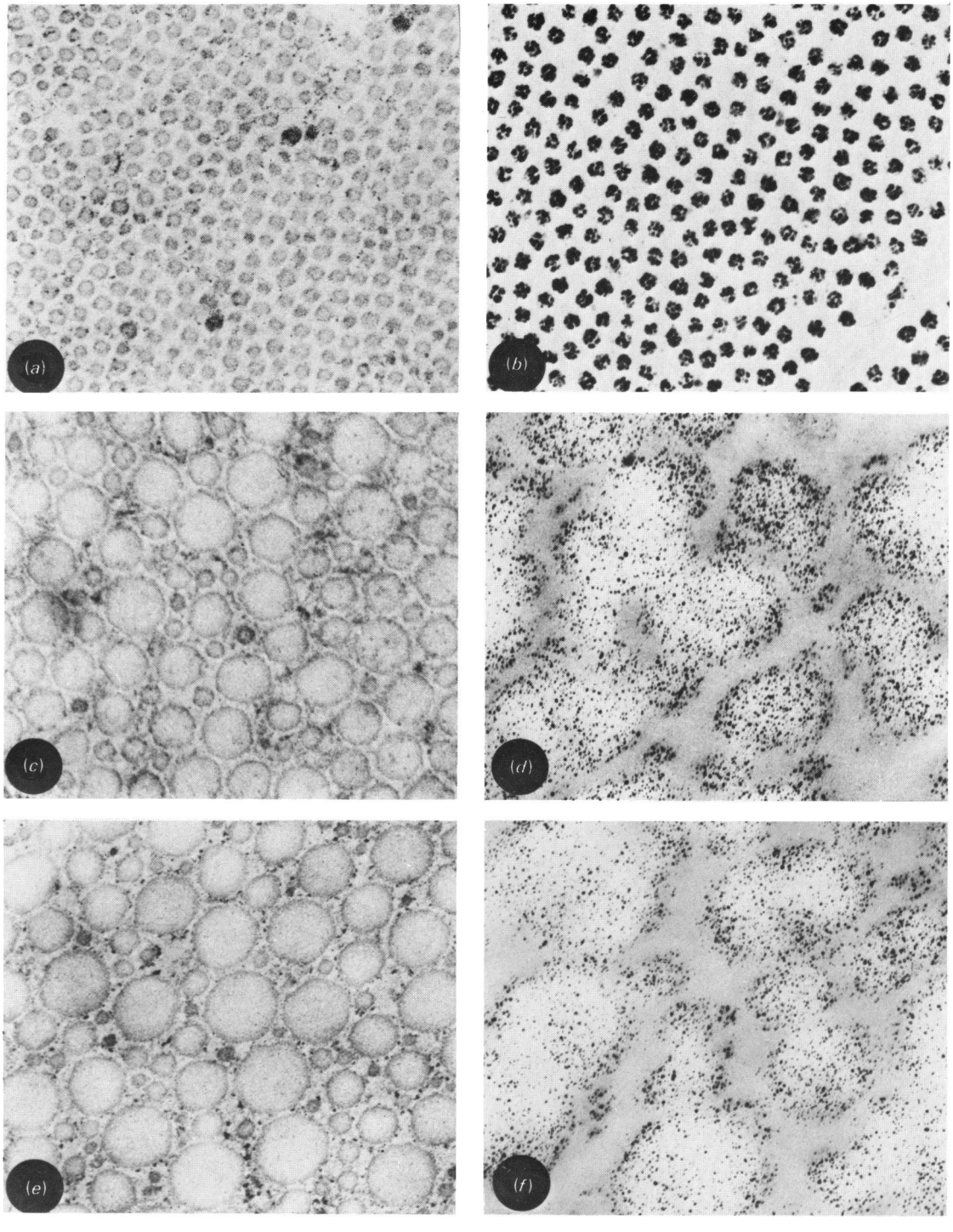


Fig. 1. *Changes with age in the content of hexosamines (a, c) and the ratio glucosamine/galactosamine (b, d), as determined on papain whole digest of rabbit Achilles tendon (a, b) and in cetylpyridinium chloride-precipitable material (c, d)*

O, Total hexosamines; ▲, galactosamine; △, glucosamine. The age is expressed as log days, before birth on the left and after birth on the right of the broken vertical line. For full experimental details, see the text.


(a)
(b)
(c)
(d)
(e)
(f)

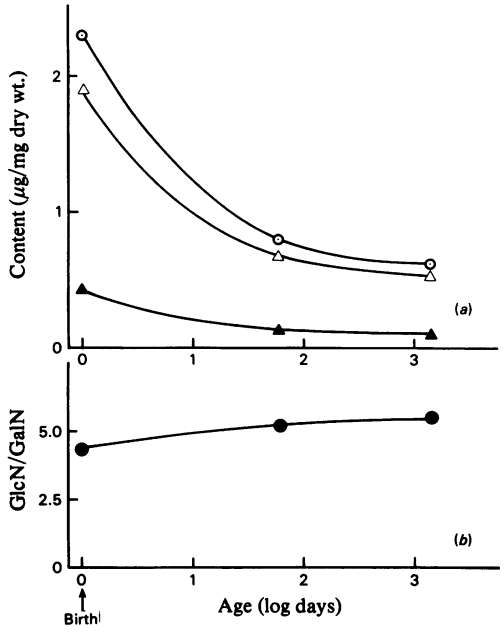


Fig. 2. *Changes with age in the content of hexosamines (a) and in the glucosamine/galactosamine ratio (b) in cetylpyridinium chloride-non-precipitable material*
○, Total hexosamines; ▲, galactosamine; △, glucosamine. In this fraction uronic acids were not detectable.

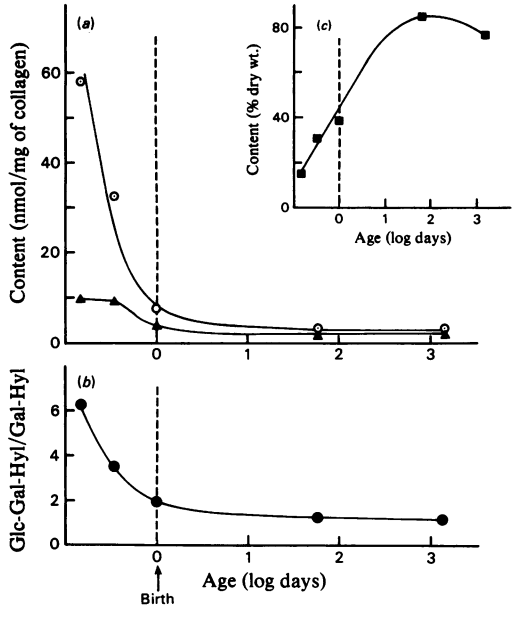


Fig. 3. *Changes with age in the content of hydroxylysyl glycosides (a), in the ratio diglycoside/monoglycoside (Glc-Gal-Hyl/Gal-Hyl) (b) and in the collagen content (c)*
(○) Glucosylgalactosylhydroxylysine; (▲) galactosylhydroxylysine. For full experimental details, see the text.

These data were in good agreement with the results of Scott *et al.* (1981*a*,*b*), who showed, in rat tail tendon, a decrease with ageing of the tissue concentration of sulphated glycosaminoglycans, with only a relative increase of dermatan sulphate.

In the 'glycoprotein fraction' (cetylpyridinium chloride-not-precipitable material from the papain total digest), a sharp decrease of hexosamine content after birth was found (Fig. 2*a*), whereas the value of the ratio glucosamine/galactosamine did not significantly change (Fig. 2*b*). In this fraction uronic acid was not detectable. Further characterization of the 'glycosaminoglycan' and 'glycoprotein' fractions from foetal rabbits could not be performed owing to the small amounts of material available.

The collagen content of the dry tendon markedly increased with the development of the tissue, during foetal growth until 2 months after birth (Fig. 3*c*), in agreement with the results of Ippolito *et al.* (1980) and with the similar data of Scott *et al.* (1981*a*,*b*). Neither electrophoresis nor amino acid analysis indicated the presence of type III collagen; also, in foetal tissues, only type I collagen was detectable.

The total content of hydroxylysyl glycosides in tendon collagen was clearly age-dependent; in fact the content markedly decreased during foetal life and, to a lower extent, after birth.

Hydroxylysyl diglycoside occurred more frequently than monoglycoside and its content was the greater the younger the animal.

This value decreased to about one-sixth of the original level during the last week of foetal life, with a further 50% decrease after birth during tissue maturation. With further ageing no significant changes did appear (Fig. 3*a*). Hydroxylysyl monoglycoside showed a similar, but less evident, decrease, and this clearly explains the behaviour of the di-/mono-glycoside ratio observed with ageing (Fig. 3*b*).

It is noteworthy that the extent of glucosylation (i.e. the hydroxylysyl di-/mono-glycoside ratio) mainly decreased as the collagen content and the fibre diameter increased.

Discussion

The periodate/silver/methenamine reaction for electron microscopy identifies neutral-sugar-containing molecules in the same way as the periodate/Schiff reaction does at light-microscopic level (De Martino & Zamboni, 1967).

Since under usual conditions proteoglycans are either periodate/Schiff-negative or only weakly positive (Scott & Harbison, 1968; Puddu & Ippolito, 1974), the absence of silver grains we observed in the

interfibrillar matrix may indicate that proteoglycans are almost periodate/silver/methenamine-negative as well. So the observed strong positivity of collagen fibrils could be mainly due both to the structural glycoproteins, which are rich in neutral sugars and are associated with the collagen in tendon matrix, and to the glucidic units linked to hydroxylysine residues in collagen molecules. The positivity of collagen fibrils to silver/methenamine decreases rapidly during maturation and parallels both the decrease in hydroxylysyl glycoside content and the decrease in structural glycoproteins. At the same time, the average diameter of collagen fibres increases, as described by several authors, during tendon maturation (Fitton-Jackson, 1956; Schwartz, 1957; Torp *et al.*, 1975; Hall, 1976; Parry & Craig, 1978; Scott *et al.*, 1981*a*,*b*). Anderson *et al.* (1977) postulated that structural glycoproteins could regulate the diameter of collagen fibrils by modulating the aggregation of tropocollagen molecules; in fact he showed that the increase in diameter is preceded by a loss of structural glycoproteins. We confirmed their results and demonstrated, in addition, a clear variation of the content of hydroxylysyl glycosides, supporting also the hypothesis of Morgan *et al.* (1970) that an inverse correlation exists between the carbohydrate content of collagen and fibril diameter.

Since Anttinen & Hulkko (1980) showed that the glucosylation of galactosylhydroxylysine in collagen seems to be rapidly completed during collagen biosynthesis, the decrease of di-/mono-glycoside ratio we have observed in tendon during foetal growth and, to a lower extent, during postnatal life, appears highly remarkable. These results suggest a conversion of diglycoside into monoglycoside occurring together with the decrease in total glycosylation, i.e. the loss of disaccharide chains by the collagen molecules. The recent demonstration of a specific glucosylgalactosylhydroxylysine glucohydrolase in mammalian tissues (Hamazaki & Hotta, 1980) seems to substain this hypothesis. However, other possibilities cannot be excluded: in a rapidly growing tissue, the new collagen formed by maturer tissue, which contains less diglycoside, would be added to that already present, producing the observed change in the proportion of diglycoside.

Therefore not only structural glycoproteins, but also the extent of glycosylation, seem able to play an important role in tropocollagen organization and in fibril growth during maturation and ageing; our data suggest that the presence of a large number of the extrusive groups of hydroxylysyl glycosides, and chiefly of the bulky disaccharide side chain of hydroxylysyl diglycoside, may delay the packing of collagen molecules and the growth of the fibrils laid down. The rapid increase of collagen content observed in foetal tendons of different ages, corresponding to the sharp decrease of the content of hydroxylysyl glycosides and of the di-/mono-glycoside ratio, seems to support this hypothesis.

Particular attention should be paid to the proteoglycan fraction of the matrix that decreases in content and varies in composition with ageing, as described by Scott *et al.* (1981*a*,*b*) in rat tail tendon. These results suggest that a correlation exists between proteoglycan modifications and collagen-fibre maturation and growth, and in this respect it is remarkable that, in the absence of significant differences in morphology, only small variations of proteoglycan composition appeared, whereas in the presence of evident modifications in size of collagen fibrils (e.g. between newborn and young animals), great variations in proteoglycan composition occurred.

It is therefore possible that the changes observed with ageing in collagen glycosylation, in glycoprotein content and in proteoglycan concentration and composition are different aspects of a single and complex process, leading to morphological and physical modifications of the tissue.

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