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Proteoglycan–collagen arrangements in developing rat tail tendon

An electron-microscopical and biochemical investigation

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1. Developing tail tendons from rats (19-day foetal to 126 days *post partum*) were examined by electron microscopy after staining for proteoglycan with a cationic copper phthalocyanin dye, Cuprolinic Blue, in a 'critical electrolyte concentration' method. Hydroxyproline was measured on papain digests of tendons, from which glycosaminoglycuronans were isolated, characterized and quantified. 2. Mean collagen fibril diameters increased more than 10-fold with age according to a sigmoid curve, the rapid growth phase 2 being during 30–90 days after conception. Fibril periodicities were considerably smaller (50–55 nm) in phases 1 and 2 than in phase 3 (>62 nm). 3. Dermatan sulphate is the main glycosaminoglycuronan in mature tendon. Chondroitin sulphate and hyaluronate preponderate in foetal tissue. 4. Proteoglycan was seen around but not inside collagen fibrils. Proteoglycan and collagen were quantified from electron micrographs. Their ratios behaved similarly to uronic acid/hydroxyproline and hyaluronate/hydroxyproline ratios, which decreased rapidly around birth, and then levelled off to a low plateau coincident with the onset of rapid growth in collagen fibril diameter. 5. Dermatan sulphate/hydroxyproline ratios suggest that the proteoglycan orthogonal array around the fibril is largely dermatan sulphate. In the foetus hyaluronate and chondroitin sulphate exceed that expected to be bound to collagen. 6. An inhibiting action of chondroitin sulphate-rich proteoglycan on fibril diameter growth is suggested. 7. The distributions of hyaluronate, chondroitin sulphate and dermatan sulphate are discussed in the light of secondary structures suggested to be present in hyaluronate and chondroitin sulphate, but not in dermatan sulphate.

Connective tissue is a shared environment created by two or more cells, which has evolved to cope with mechanical stresses associated with movement and the maintenance of shape, while permitting metabolites to flow to and from cells. Protein fibres (e.g. collagen) cope with tensile stresses, whereas compressive forces are opposed by polymers (e.g. proteoglycans), dissolved in the interfibrillar fluids, which tend to 'inflate' the fibre network. The soluble polymer domain is the channel through which small molecules can diffuse freely (Scott, 1975, 1979). This model of a fibre-reinforced composite material appears to be valid throughout much of biology. The details of the relationship between fibres and soluble polymer determine the capacity of the tissue to function in its own way. In tendon, there is clear evidence of specific interactions between collagen and proteoglycan, whereas interactions in cartilage are probably less specific, involving entrapment and excluded volume (Scott, 1975, 1980). In the earliest stage of fibrillogenesis, results obtained *in vitro* suggest that glycosaminoglycans influence fibre nucleation and growth (Öbrink, 1973). During maturation of tendon there is an increase in collagen fibril diameter (Fitton-Jackson, 1956; Torp *et al.*, 1975; Parry & Craig, 1978), but nothing is known of the influence of proteoglycan on this process.

Rat tail tendon is a simple easily accessible tissue consisting mainly of type I collagen with a little proteoglycan. It offers excellent prospects for studying the spatial and temporal relationships between proteoglycan and collagen, by electron microscopy and biochemical analysis. This has hitherto not been possible, because electron-microscopical–histochemical techniques for localizing and identifying proteoglycans were inadequate. Recently, new reagents and techniques based on the 'critical-electrolyte-concentration' principle have been introduced

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(Scott, 1975) and applied to rat rail tendon (Scott, 1980). Foetal and adult tissue showed strong similarities. Seen in longitudinal section, proteoglycan filaments were orthogonally arrayed about the fibril, with spacings between the horizontal components (i.e. at right-angles to the fibril axis) equal to those of the collagen-fibre banding periodicity (60–65 nm in mature tendons).

The present paper seeks to complete a three-dimensional picture of the perifibrillar arrangement of proteoglycan by examining transverse sections of tendons. Since little was known about the biochemistry of the acidic glycosaminoglycans of young rat tail tendon, we have examined this aspect both qualitatively and quantitatively, as a function of age, and have correlated the findings with electron-microscopical histochemistry.

The results are used in constructing a sequence of events starting from tropocollagen and leading to large-diameter collagen fibrils. An inhibiting action of chondroitin sulphate-rich proteoglycan on fibril diameter growth is postulated.

The electron-microscopical findings show specific relationships between proteoglycan and collagen fibrils. Specific interactions are profoundly affected by secondary and higher-order structures within the participants themselves. For example, the assembly of soluble tropocollagen into insoluble fibrils multiplies the possibilities and transforms the situation. Secondary structures in glycosaminoglycuronans must affect interactions with other molecules. Our results are discussed from the standpoint of hydrogen-bonded structures suggested to be present in hyaluronate and chondroitin 4-sulphate but not in dermatan sulphate (Scott & Tigwell 1978; Scott & Heatley, 1979).

Some of these data have been preliminarily communicated (Scott & Orford, 1980).

Materials and methods

Electron-microscopy materials

Tendons taken from half-way along the tail of rats of age 25, 30, 33, 40, 45 and 28 days after conception were teased to individual fibres in iso-osmotic saline (0.9% NaCl). The 90-day and 149-day tendon fibres were too large to be embedded whole, and were therefore cut into the smallest pieces that could be handled conveniently. The 19-day, 20-day or 21-day foetal rat tails were embedded whole in lengths of approx. 1 mm.

Cuprolinic Blue (dye b; Scott, 1980) was from BDH Chemicals, Poole, Dorset, U.K.

Materials for electron microscopy were from Agar Aids, Stanstead, Essex, U.K.

Biochemistry materials

Tendons from rat tails 30 days to 149 days after conception were dissected out. Two to ten rats provided material for each set of analyses. Tendons from 19-day foetal (one to three litters, 10–16 foetuses/litter) to 25-day-post-conception rats were dissected out under a low-power dissecting microscope. Foetal 'tendon' was the material left after the tail skin and cartilaginous core had been removed. Tendons were immersed for 20 h in several changes of acetone, then dried in air for 2–3 h to constant weight.

Papain (EC 3.4.22.2) (2 × recrystallized, in suspension) was from BDH Chemicals.

Electron-microscopical methods

Methods were essentially those of Scott (1980), except that sodium tungstate was included in the post-staining rinses ('en bloc' staining) and the use of sodium tungstate on plastic sections could be dispensed with.

Tendon was stained overnight at room temperature in Cuprolinic Blue at 0.05% in 25 mM-sodium acetate buffer, pH 5,7, containing $MgCl_2$ at 0.1 M and glutaraldehyde at 2.5% (w/v). The tissue was rinsed three times in buffer with glutaraldehyde and $MgCl_2$ of the same concentrations as in the staining solution for a total time of 15 min. It was washed three times in aq. 0.5% sodium tungstate for a total of 15 min, then in 0.5% sodium tungstate in 50% (v/v) ethanol, dehydrated and embedded in Taab epoxy resin in polythene capsules.

Sections cut from the blocks were examined in a Philips 301 electron microscope. A grating replica (2160 lines/mm) was used to check magnification, and all measurements were referred back to this standard.

Quantitative analysis of electron micrographs

Results were obtained from at least two blocks from each of two rats at all ages, except for the 149-day-post-conception (126 days *post partum*) investigation, where only one rat was used.

Collagen fibril diameter. For each age, ten micrographs showing regions of clearly defined collagen fibrils were selected. Twenty fibril diameters were measured from each micrograph. The mean (=sum of all diameters/total number of diameters) and standard error of the mean were calculated.

Collagen banding distance and proteoglycan spacing. The collagen banding distance was estimated for a single fibril on each micrograph. A length of fibril corresponding to between 10 and 20 tungstate-stained bands was measured, and the separations between bands were calculated.

The spacing of proteoglycan filaments was estimated similarly, by using the same fibrils from the same micrographs.

Ratio of proteoglycan to collagen. Stained proteo-

glycan appears as electron-dense filaments outside the electron-lucent collagen fibrils (Plates 1 and 2). The proteoglycan/collagen ratio equals the following:

$$\frac{\text{Total length of proteoglycan filaments in an area on an electron micrograph}}{\text{Total cross-sectional area of collagen fibrils in the same area}}$$

At least ten micrographs from each of at least three different blocks were examined for each age. The mean and standard error of the mean for each age were calculated.

Biochemical methods

Glycosaminoglycuronans were prepared from tendons essentially by the method of Scott (1960). Digestion by papain (5 μl of suspension/ml of digest) in 0.3 M-NaCl/0.1 M-sodium phosphate buffer, pH 6.4, containing 5 mM-cysteine hydrochloride and 0.2 M-Na_2EDTA at 65°C was followed by dilution with 4 vol. of water in the presence of cetylpyridinium chloride. The cetylpyridinium precipitates were washed with 60 mM-NaCl and water and dissolved in 66% (v/v) propan-1-ol. The sodium salts of the polyanions were precipitated with saturated ethanolic sodium acetate (10 vol.). The polyanions were fractionated with cetylpyridium at 0.15 M-Na_2SO_4, to give 'hyaluronate' in the supernatant and 'sulphated polysaccharides' in the precipitates (Scott, 1960). Cetylpyridinium hyaluronate was recovered from the supernatant after dilution with water to 60 mM-Na_2SO_4, followed by centrifugation and washing with water. Cetylpyridinium complexes were converted via 66% propan-1-ol into sodium salts as described above. 'Sulphated polysaccharides' were fractionated by the addition of ethanol to aqueous solutions containing 5% (w/v) calcium acetate and 0.5 M-acetic acid (Meyer *et al.*, 1956). At 13.8% (v/v) ethanol nucleic acid was recovered, then 'dermatan sulphate' at 23.5% (v/v) ethanol and 'chondroitin sulphate' at 80% (v/v) ethanol. Precipitates were dissolved with the aid of a few drops of 0.1 M-Na_2EDTA, and the sodium salts were recovered by the addition of 10 vol. of saturated ethanolic sodium acetate. Electrophoreses were in 0.1 M-barium acetate or 0.1 M-HCl (Wessler, 1970) or in 0.1 M-calcium acetate on cellulose acetate strips.

Polyanions were stained with Alcian Blue, and after dissolution of the cellulose acetate membrane in dimethyl sulphoxide (Newton *et al.*, 1974) they were quantified as their Alcian Blue complexes, with chondroitin 4-sulphate and dermatan sulphate as standards.

Uronic acid was measured by the Dische carbazole method, with incorporation of borate (Bitter & Muir, 1962) in a scaled-down (0–5.0 μg) version with 2 cm semi-micro cuvettes and total volumes of 1.08 ml. 'Dermatan sulphate' was estimated in the ethanolic-calcium acetate fractions by the method of Di Ferrante *et al.* (1971), with chondroitin 4-sulphate and dermatan sulphate as standards. Hydroxyproline was determined by the method of Woessner (1961).

Results

Electron microscopy

Cross-sections of tendons of animals of various ages are illustrated in Plates 1 and 2. The distributions of fibril diameters for each age are plotted in Fig. 1. Extreme values are 12 and 511 nm.

In every young tissue, the distribution of fibril diameters was narrow. With increasing age, the distribution widened. The oldest tissues contained many irregularly shaped large fibrils, which may have formed from the fusion of small fibrils (Plate 2*b*). The mean diameter of the fibrils increased with age and the points lie on a smooth sigmoid curve (Fig. 2).

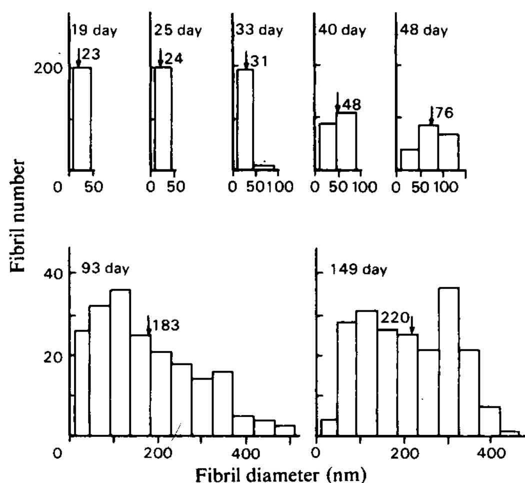


Fig. 1. *Changes with age in collagen-fibril diameter distributions in rat tail tendon*

For full experimental details see the text and the legend to Fig. 2. Mean diameters (nm) are indicated by vertical arrows (↓). The distribution of fibre diameters in tendon is narrow in foetal animals and immediately *post partum*. In the weeks after birth the distribution broadens to include fibrils 15 times the diameter of foetal fibres.

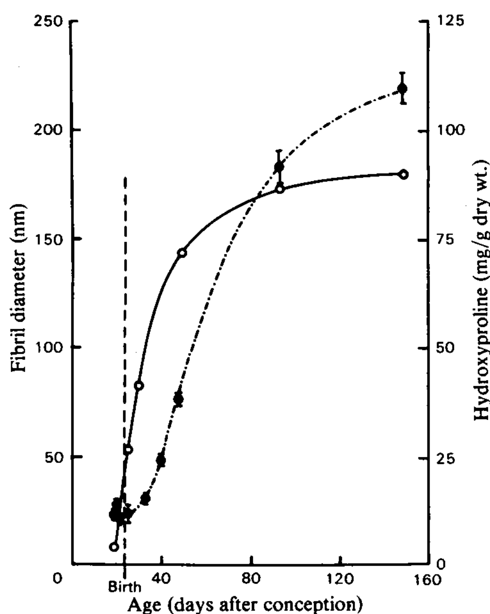


Fig. 2. *Changes with age in mean collagen fibril diameter* (●) *and hydroxyproline content* (○) *in rat tail tendon*
Diameters of 200 fibrils (bars represent S.E.M.) were measured on ten micrographs, from at least two blocks from each of two rats at each age. Hydroxyproline was assayed on papain-digested tendon, after acid hydrolysis. For full experimental details see the text.

In longitudinal section the distribution of the proteoglycan filaments along the collagen fibrils was related to the longitudinal banding pattern of the collagen (Scott, 1980; Fig. 4). In transverse section proteoglycan was seen only outside the collagen fibrils (Plates 1 and 2). Filaments sometimes appeared to radiate out from the fibrils and may have connected them. In most cases the filaments lay tangentially to, or curved around the circumference of, the fibrils. In materials from very young animals a single proteoglycan filament often encompassed more than one collagen fibril, but in material from older animals a number of filaments were associated with a single collagen fibril.

There was a decrease with increasing age in the ratio of proteoglycan to collagen (Fig. 3*a*), measured from electron micrographs, that was most rapid between late foetal age and 48 days after conception, levelling off in the adult. Tissue stained en bloc with sodium tungstate according to the modification introduced in the present work showed a more-or-less constant higher ratio of proteoglycan to collagen at all ages than did tissue post-stained with sodium tungstate (Scott, 1980), but the shape of the plot was the same (results not shown).

Biochemistry

Papain-digested tendon polyanions contained sulphated glycosaminoglycans and nucleic acids, which were precipitated by cetylpyridinium at 0.15 M-Na_2SO_4. The supernatant deposited predominantly cetylpyridinium hyaluronate on dilution to 60 mM-Na_2SO_4. After conversion into sodium salts, electrophoresis in 0.1 M-HCl (Wessler, 1970) confirmed the presence of sulphated polyanion in the first fraction and its absence from the second. Fractional precipitation of the first fraction in the presence of calcium acetate by ethanol at 13.8%, 23.5% and 80% (v/v) produced three further fractions. The 13.8%-ethanolic calcium acetate fractions contained nucleic acids, as shown by the very high absorption at 260 nm, but negligible amounts of uronic acid. The 13.8–23.5%-ethanolic-calcium acetate fractions were largely dermatan sulphate, and the 23.5–80%-ethanolic fractions mainly chondroitin sulphate, as shown by electrophoresis in 0.1 M-barium acetate or -calcium acetate. The 'dermatan sulphate' band extended forward part way towards the chondroitin sulphate position, compared with standard dermatan sulphate.

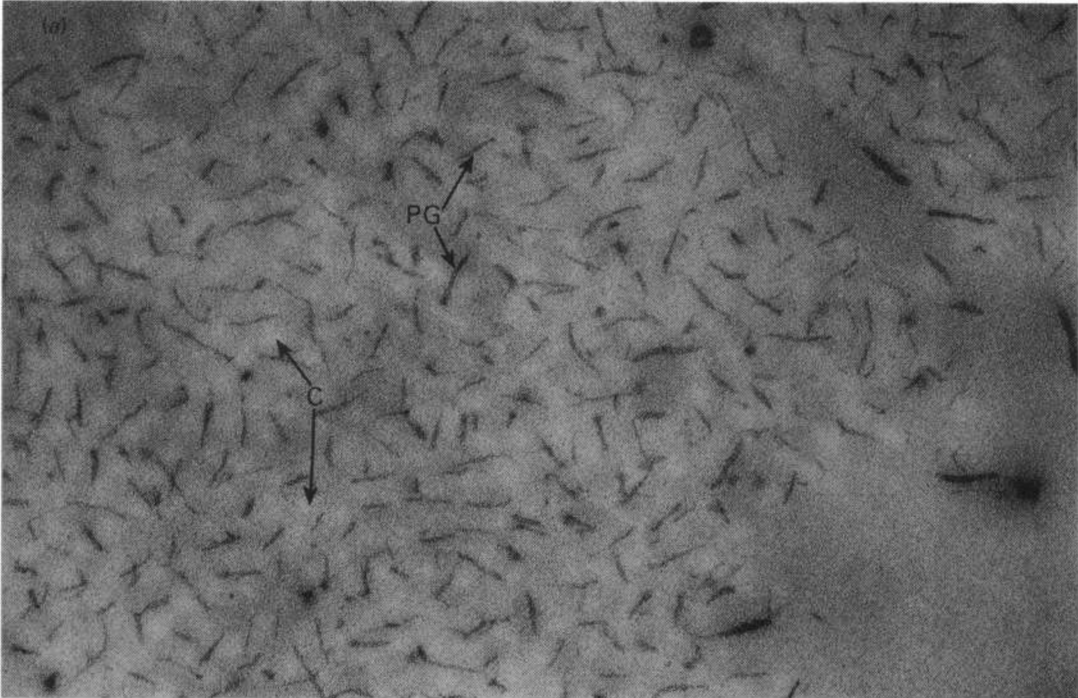
On electrophoresis a single sulphated polyanion was observed in 0.1 M-barium acetate in the 19-day-post-conception material, moving similarly to but slightly more slowly than standard chondroitin 4-sulphate. The main component of mature tendon sulphated glycosaminoglycan was dermatan sulphate. The foetal chondroitin sulphate moved appreciably more slowly than did standard chondroitin sulphate on electrophoresis in 0.1 M-HCl, whereas the mobility of tendon dermatan sulphate was very similar to that of standard chondroitin sulphate or dermatan sulphate.

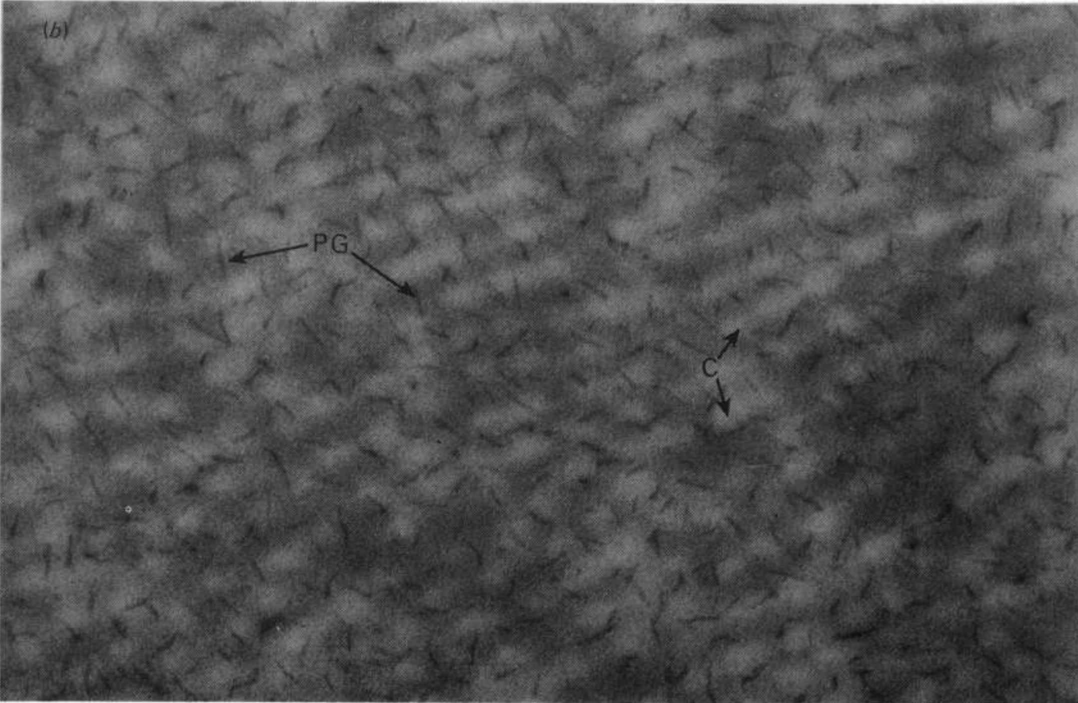
The total nucleic acid [mg/g dry wt. of tissue (calculated from the u.v. absorption at 260 nm of

EXPLANATION OF PLATES 1 AND 2

Transverse sections of rat tail tendon

1(*a*) 21 days after conception; 1(*b*) 30 days after conception; 2(*a*) 45 days after conception; 2(*b*) 93 days after conception. Magnification ×106000. The sections were stained with Cuprolinic Blue (0.05%) in 0.1 M-$MgCl_2$/25 mM-sodium acetate buffer, pH 5.7, containing 2.5% glutaraldehyde, See the text for details. Collagen fibrils (C) are approximately circular in cross-section. Proteoglycans (PG) are deeply stained filaments closely associated with the outside of the collagen fibrils. Proteoglycans are not visible inside the fibrils. The sizes and the range of sizes of collagen fibrils increase with age. Irregularly shaped fibrils (I) may result from fusion (F) of adjacent fibrils. For full experimental details see the text.


(a)
PG
C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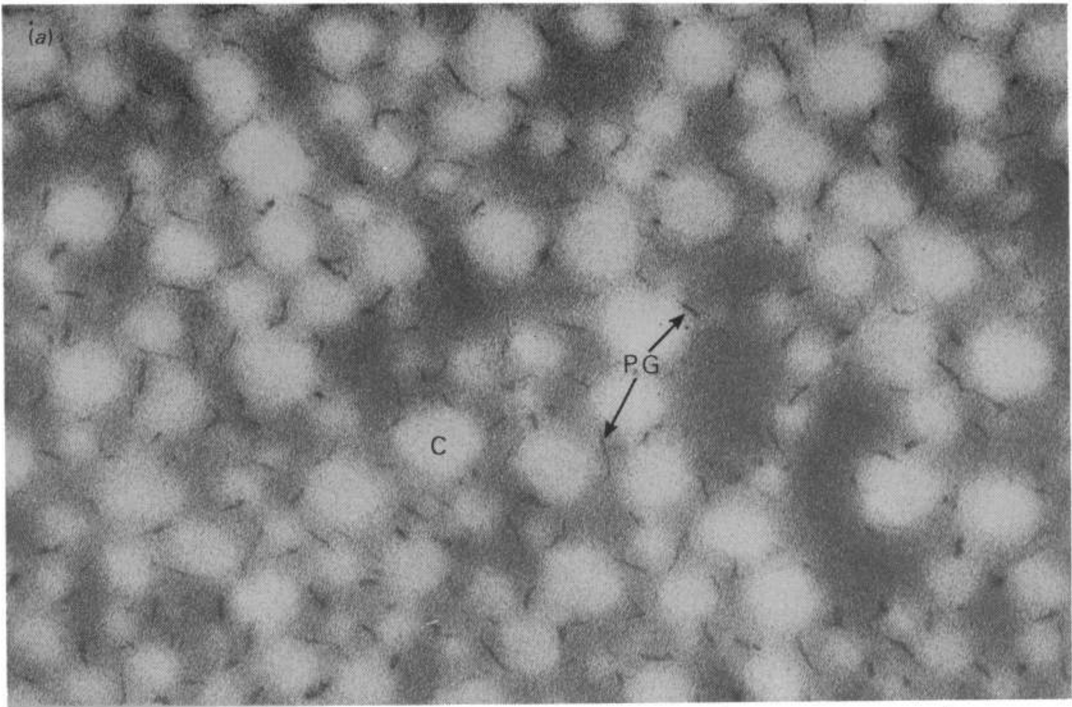

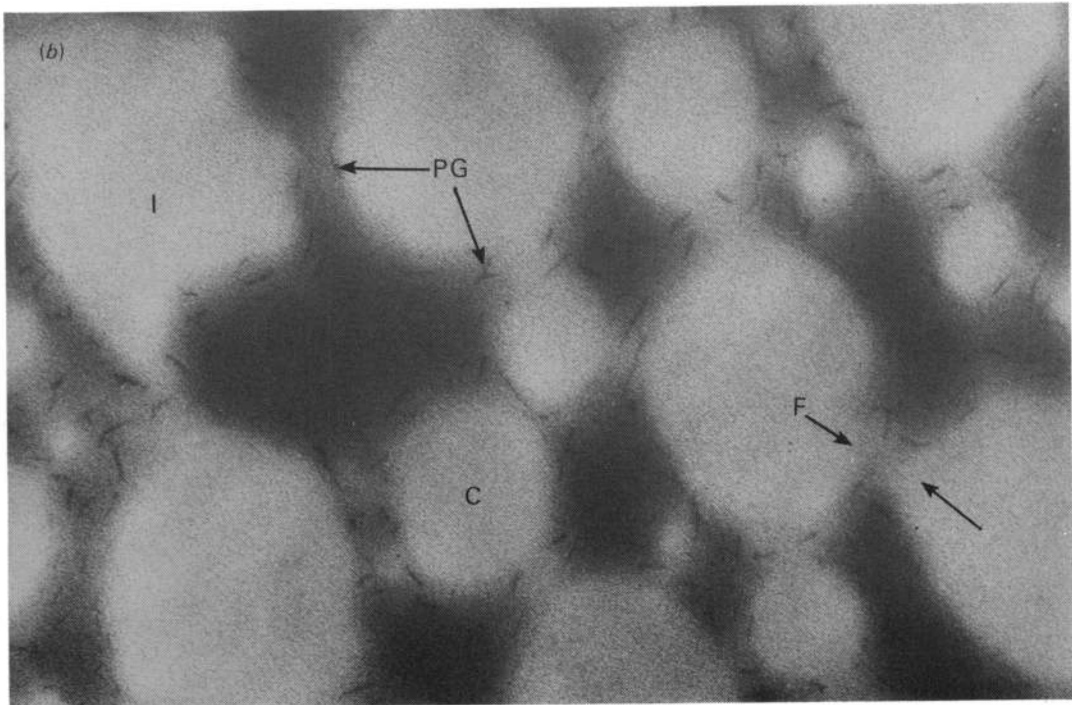

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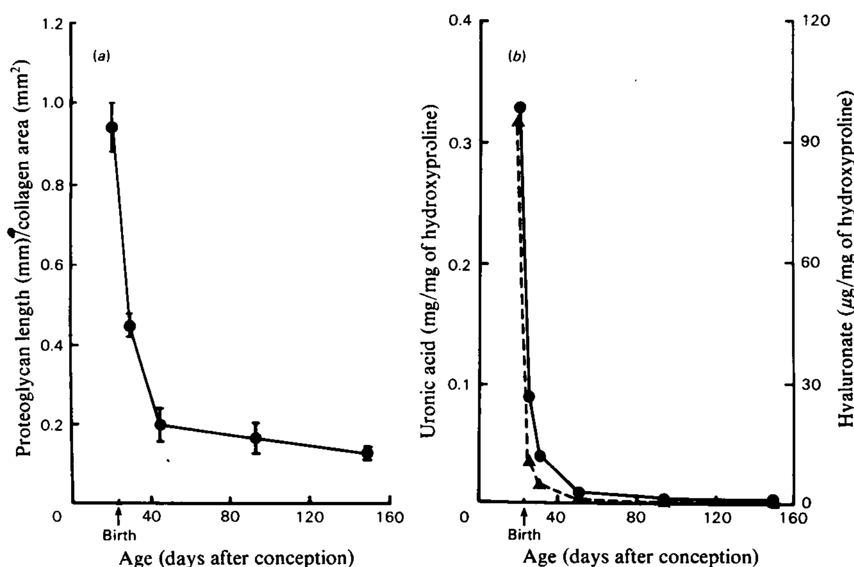

Fig. 3. *Changes with age in the ratio of proteoglycans to collagen in rat tail tendon*
(*a*) From electron micrographs: total length of proteoglycan filaments per unit collagen cross-sectional area. (*b*) From biochemical analyses, performed on papain-digested tendon: ●, uronic acid content (mg/mg of hydroxyproline); ▲, hyaluronate content (μg/mg of hydroxyproline). For full experimental details see the text.

Table 1. *Chemical analyses of rat-tail tendon*

Anhydrohexuronic acid was measured on the cetylpyridinium fraction insoluble at 0.15 M-Na_2SO_4. This fraction contains sulphated glycosaminoglycuronans. Total sulphated glycosaminoglycan in this fraction (column 4) was quantified as the Alcian Blue-complexed polyanion migrating during electrophoresis on cellulose acetate strips (Newton *et al.*, 1974). 'Hyaluronate', isolated as the cetylpyridinium fraction soluble at 0.15 M-Na_2SO_4 but insoluble at 60 mM-Na_2SO_4, was quantified as the Alcian Blue-complexed polyanion moving as hyaluronate on cellulose acetate strips (Newton *et al.*, 1974). Dermatan sulphate was determined either (*a*) by the method of Di Ferrante *et al.* (1971) (column 5) or (*b*) as the Alcian Blue-complexed polyanion migrating as dermatan sulphate on electrophoresis (column 6). For full experimental details see the text.

Age of sample (days after conception; birth = 23 days)		Composition (mg/g dry wt. of tendon)					
		Hydroxyproline	Anhydrohexuronic acid	Total sulphated glycosaminoglycan	Dermatan sulphate		Hyaluronate
					(*a*)	(*b*)	
Foetal	19	3.8	1.23		<0.3		0.36
Post partum	25	26.5	1.9	4.1	2.6	1.5	0.27
	30	41.0	1.6	3.03	3.0	2.1	0.14
	49	71.4	[illegible]	1.9		1.25	0.055
	[illegible]	[illegible]	[illegible]	1.2		0.9	0.03
	149	89.7	0.3	0.65		0.6	0.013

the 13.8%-ethanolic fraction)] decreased markedly from 42 at 19 days to 34 at 25 days, 23 at 30 days and 2.3 at 93 days, in parallel with the numbers of cells visible in the low-magnification micrographs.

Hydroxyproline per mg dry wt. of tendon increased sharply and steadily from 19 days to about 50 days after conception, and more slowly thereafter, levelling off at about 90 days after conception (Fig. 2).

Two independent methods of measuring glycosaminoglycuronan, i.e. assay of uronic acid or of the polysaccharide–Alcian Blue complexes eluted from electrophoretic strips, showed similar trends. The ratios of sulphated glycosaminoglycuronan or hyaluronate to hydroxyproline declined sharply immediately around birth, and decreased more slowly thereafter (Fig. 3*b*). Their tissue concentrations diminished with age. Electrophoretic and

colorimetric assays showed an increase with age (up to day 30 after conception) in the tissue concentration of dermatan sulphate, and a decline thereafter. There was a decline in the ratio of dermatan sulphate to hydroxyproline with age, but an increase in the percentage of dermatan sulphate in the sulphated glycosaminoglycuronan fraction (Table 1).

Discussion

Dermatan sulphate and chondroitin sulphate have long been recognized as the main glycosaminoglycuronan components of mature bovine tendon (Meyer *et al.*, 1956). More recently, the corresponding proteoglycans have been prepared (Anderson, 1975). There were no corresponding data on rat tail tendon, particularly from the very young animal, where only minute amounts of tissue are available (about 300 μg dry wt. per 19-day foetus). We therefore characterized the main polyanions at ages 19–149 days after conception. Papain digestion was followed by cetylpyridinium fractionation into (1) the sulphated polyanions and (2) 'hyaluronate' (Scott, 1960). The high mobility of fraction 1 on electrophoresis in 0.1 M-HCl (Wessler, 1970) confirmed that it was indeed sulphated, whereas the slow-moving fraction 2 was not. Electrophoresis in 0.1 M-barium acetate or -calcium acetate showed fraction 2 to be mainly hyaluronate, with a minor component moving somewhat faster, visible only with material obtained 25 and 30 days after conception. Fraction 1 from mature rat tail tendon migrated largely as dermatan sulphate. However, the foetal sulphated polyanion was almost exclusively chondroitin sulphate, which electrophoresis in 0.1 M-HCl showed to be undersulphated. During maturation, the proportion of dermatan sulphate to chondroitin sulphate increased sharply from 25 to 48 days after conception (Table 1). Confirmation of the identity of the two components came (*a*) from fractionation with ethanolic calcium acetate by the procedure of Meyer *et al.* (1956), and (*b*) by the high reactivity of the 13.8–23.5%-ethanolic fraction in the Di Ferrante *et al.* (1971) method for dermatan sulphate, plus the absence of reactivity in the 23.5–80%-ethanolic fraction. Less than 2% of the standard dermatan sulphate colour yield was given by standard chondroitin sulphate.

Further characterization of the chondroitin sulphate fraction, or of the minor components, could not be performed because of the small amounts of material available.

Comparison of electron microscopy with biochemical analyses

Uronic acid assay is therefore an acceptable index of tendon sulphated proteoglycan. Electrophoretic-strip assays provide relatively specific measurements of hyaluronate. dermatan sulphate and chondroitin sulphate contents. The colorimetric assay of dermatan sulphate (Di Ferrante *et al.*, 1971), based on the observation (Scott, 1968) that it is oxidized to aldehydes by periodate much more rapidly than is chondroitin sulphate and consequently gives far more colour with Schiff reagent, is less specific, since heparan sulphate and other glucuronic acid-containing glycosaminoglycans are also oxidized rapidly (Scott & Tigwell, 1978). Nevertheless, the results are in accord with the electrophoretic and uronic acid assays, probably because preliminary fractionation produced materials suited to the method, and heparan sulphate is not an important constituent of tendon.

The electron micrographs (Plates 1 and 2) show proteoglycan filaments of variable length. Their total length, assessed from many micrographs, should be a guide to the total tissue proteoglycan. The cross-sectional area of collagen fibrils is a function of the collagen content, given a constant diameter throughout the fibril length. Thus the ratio of total proteoglycan filament lengths to total collagen cross-sectional areas, taken from a sufficient number of micrographs, ought to be related to the uronic acid/hydroxyproline ratio, determined in parallel. Changes during development of rat tail are large, providing a good test of the correlation over a wide range of values.

The 'vertical' elements (parallel to the fibrils) in the orthogonal array (Scott, 1980) are underestimated, since they appear in cross-section as dots. Preliminary results suggest that there are proportionately more vertical components in the very young tissue, requiring a greater (but not large) correction to the estimated proteoglycan length.

The assumption that equal lengths of proteoglycan filament taken from tendons of different ages can be equated with the same amount of uronic acid cannot be completely true. The qualitative change from chondroitin sulphate-rich proteoglycan to dermatan sulphate-rich proteoglycan almost certainly implies a decrease in uronic acid contents. Anderson (1975, and personal communication) found that calf achilles-tendon dermatan sulphate-rich proteoglycan, in common with similar preparations from skin (Öbrink, 1973), contained much less uronic acid and more protein (50%, w/w) than did tendon chondroitin sulphate-rich proteoglycan, which is akin to cartilage proteoglycan, with 10% (w/w) protein. Nevertheless, since biochemical and electron-microscopical estimates show identical trends with age (Fig. 3), we conclude that the histochemical bases are valid. The data relating the proteoglycan/collagen ratios from electron microscopy to those from biochemistry are as would be expected from the above considerations, with the

total uronic acid in foetal tendon being under-estimated from the micrographs.

Relationship of glycosaminoglycuronans to collagen

Neither in longitudinal sections nor in cross-sections is proteoglycan visible within the fibril. Proteoglycan is seen at the periphery of the fibril as an orthogonal array. The horizontal components of the array are spaced apart by a constant distance, always equal to that of the collagen-fibril banding distance, even though this varies considerably (Fig. 4). A special relationship between the proteoglycan and periodic repeating structures along the collagen fibril is strongly implied (Scott, 1980). The ratio of proteoglycan to collagen should be in the ratio of the circumference ($2\pi r$) to the cross-sectional area of the fibril (πr^2), i.e. r^{-1}. Thus the finer the fibrils the greater the content of proteoglycan per weight of collagen. The data show that, during development, the ratio of dermatan sulphate to hydroxyproline does decrease as the fibril cross-sectional area increases. For the ratio of uronic acid to hydroxyproline or of hyaluronate to hydroxyproline, the correlation is less clear, possibly because there is a large excess of uronic acid or hyaluronate in very young tendon.

It is relevant that Gillard *et al.* (1977) found an increased concentration of dermatan sulphate in the sesamoid portion of rabbit flexor tendon digitorum profoundis compared with the rest of the tendon, and our preliminary investigations (J. E. Scott, C. R. Orford & E. M. Hughes, unpublished work) show that the sesamoid region has the thinnest fibrils. The corollary, that coarser fibrils are associated with less dermatan sulphate per unit weight of collagen, can be inferred (*a*) from the results obtained by Nakamura & Nagai (1980), who found a decrease in dermatan sulphate in the skin of chicks after hatching, although the amount of collagen was increasing, and (*b*) from the observation (Tajima & Nagai, 1980) that the upper dermis of calf skin, containing 'fine' fibrils, has twice the dermatan sulphate/collagen ratio of the lower dermis, containing 'coarse' fibrils.

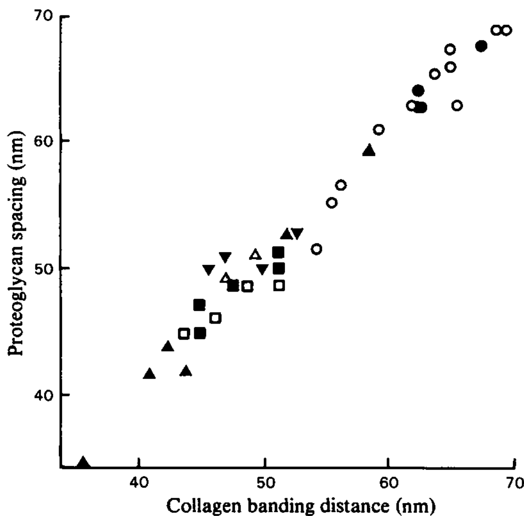

Fig. 4. *Correlation of collagen axial banding periodicities and proteoglycan spacings in developing rat tail tendon*

●, 149 days after conception; ○, 93 days after conception; ■, 48 days after conception; □, 45 days after conception; ▲, 40 days after conception; △, 33 days after conception; ▼, 25 days after conception. Each point represents data from one micrograph, i.e. the mean of 10 to 20 separations along a single fibril. For full experimental details see the text.

Hoffman *et al.* (1957) and Loewi & Meyer (1958) suggested that dermatan sulphate is associated with coarse collagen fibres. It seems rather that dermatan sulphate binds specifically to collagen fibrils of all diameters, but this is not obvious in tissues containing fine fibrils, which characteristically are rich in chondroitin sulphate or hyaluronate. Tissues rich in coarse fibrils contain less condroitin sulphate (Loewi & Meyer, 1958), and the contribution of dermatan sulphate to the analyses is consequently more striking.

The spacing between the proteoglycan filaments in young tissue is much diminished (from 62 nm to less than 50 nm), compared with adult tissue (Fig. 4). Either (*a*) the collagen-fibre periodicity is different from that in adult, with about 50 nm (instead of 62 nm) repeat distances, and/or (*b*) there is more than one site per normal collagen repeat distance that binds proteoglycan. We observed collagen periodicities of 45–55 nm in young tendon (Fig. 4), and Gillard *et al.* (1977) reported collagen repeat distances in the sesamoid portion of rabbit extensor tendon of 53 nm. Parry & Craig (1978) noted an increase of about 10% in the periodicity of stretched, fixed and stained rat tail tendon from birth to 13 months. Jakus (1954) and Smith & Frame (1969) observed 55 nm spacings in corneal collagen fibrils.

Possibility (*a*) above is therefore likely, and, if so, alignments of groupings on adjacent collagen molecules should differ in young as compared with mature collagen. Young collagen will therefore offer different sequences of interacting groups with which proteoglycans could combine. It is noteworthy that the decreases to 53 nm in sesamoid tendon and in rat tail tendon parallel a markedly higher concentration of chondroitin sulphate than that in 'normal' or 'mature' tendon, and that the low value for the spacing in cornea is also associated with a high concentration of chondroitin sulphate (Anseth & Laurent, 1961). Conceivably, the change from 53 nm to 62 nm spacing brings about a re-alignment of sites

from a sequence favourable to chondroitin proteoglycan binding to one less suitable.

High concentrations of chondroitin sulphate are associated with fine collagen fibrils in several tissues, e.g. cornea and the pericellular environment of chondrocytes (Stockwell, 1979), as well as in young rat tail tendon. Embryonic chick skin, in which chondroitin sulphate content is high (Nakamura & Nagai, 1980), may be another instance. Fibril diameters were not reported, but they are presumably small.

Fibril maturation

The increase in fibril diameter during development follows a sigmoid curve (Fig. 2), with a phase (1) *in utero* and immediately *post partum* during which the fibril remains thin (23 nm), followed by a phase (2) of rapidly increasing diameter, and then a phase (3) of slow or negligible expansion. The results obtained by Parry & Craig (1978) and by Torp *et al.* (1975), which cover our phases 2 and 3, are quantitatively in fair agreement with ours. They did not examine foetal tail tendon, so that phase 1 has not been observed previously.

It is particularly striking that the onset of phase 2 (rapid growth) coincides with completion (at about 40 days after conception) of the sharp decline in proteoglycan/collagen ratio (Fig. 3). By far the greater part of this decline is due to loss of chondroitin sulphate (Table 1). The hyaluronate/hydroxyproline ratio declines in parallel (Fig. 3*b*). Thus the onset of phase 2 may be critically dependent on the decrease to low concentrations of chondroitin sulphate-rich proteoglycan and hyaluronate. This observation, and the association (discussed above) of chondroitin sulphate with thin fibrils, may be causally related, i.e. if a sufficient concentration of chondroitin sulphate-rich protoglycan were maintained in the tissue the outcome would be a population of thin collagen fibres.

The results and conclusions given in the present paper contribute to the following view of fibrillogenesis and fibre maturation in rat tail tendon, and probably in other tendons and connective tissues.

Phase 1 (*up to day 40 after conception*). Tropocollagen interacts with dermatan sulphate-rich proteoglycan either as part of, or immediately after, the process of aggregation to form microfibrils. The microfibrils have a decreased axial periodicity of 53 nm. The environment is rich in chondroitin sulphate-rich proteoglycan (and relatively so in hyaluronate, although less so by at least an order of magnitude). Collagen synthesis leads to the formation of increased numbers of thin fibrils, rather than to the growth in diameter of established fibrils (Fig. 2). Each fibril is associated with dermatan sulphate-rich proteoglycan, specifically located and of fixed stoicheiometry.

Phase 2 (*from day 40 to approx. day 120 after conception*). Early in this phase, concentrations of chondroitin sulphate-rich proteoglycan and hyaluronate in the perifibrillar environment are decreased to a critical value (Fig. 3), at which addition of collagen to established fibrils occurs faster than does the laying down of new fibrils. The distribution of fibril diameters broadens (Fig. 2). The mean fibril diameter increases rapidly (Fig. 2). The axial periodicity does not increase. The ratio of dermatan sulphate to fibril surface area remains constant, but the tissue concentration of dermatan sulphate diminishes (Table 1).

Phase 3 (*day 120 after conception onwards*). Fibril diameter growth slows down considerably, probably taking place by fusion of existing fibrils (Plates 1 and 2) as well as by accretion of new collagen. The axial periodicity increases to around 62 nm or more.

The sequence of chondroitin sulphate and hyaluronate decrease, and concurrent dermatan sulphate increase (end of phase 1, beginning of phase 2), has been observed in developing embryonic chick skin (Nakamura & Nagai, 1980). Fibre diameters were not measured, but increases in collagen content compatible with the above proposals were observed.

It has been suggested (Scott & Tigwell, 1978) that hyaluronate and chondroitin sulphate differ from dermatan sulphate in being able to form inter-residue hydrogen-bonded sequences along the carbohydrate chain, which stiffen them. Chondroitin sulphate and hyaluronate are thus particularly suited to occupying and maintaining large domains in solution against deforming pressures. The creation of a 'buffer volume' between the thin fibres that serves to keep them well separated and relatively free of anastomosis may be a manifestation of this property. As a corollary, dermatan sulphate has more capacity to form intermolecular liaisons, since it is not involved in intramolecular bonding to the same extent. Its role in binding specifically to collagen may bee aspect of this capacity (Scott, 1980; Meader *et al.*, 1980).

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