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Dermatan sulphate-rich proteoglycan associates with rat tail-tendon collagen at the d band in the gap region

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Rat tail tendon was stained with a cationic phthalocyanin dye, Cupromeronic Blue, in a 'critical-electrolyte-concentration' method [Scott (1980) *Biochem. J.* **187**, 887–891] specifically to demonstrate proteoglycan by electron microscopy. Hyaluronidase digestion in the presence of proteinase inhibitors corroborated the results. Collagen was stained with uranyl acetate and/or phosphotungstic acid to demonstrate the banding pattern a–e in the D period. Proteoglycan was distributed about the collagen fibrils in an orthogonal array, the transverse elements of which were located almost exclusively at the d band, in the gap zone. The proteoglycan may inhibit (1) fibril radial growth by accretion of collagen molecules or fibril fusion, through interference with cross-linking, and (2) calcification by occupying the holes in the gap region later to be filled with hydroxyapatite.

It has been speculated frequently that relationships between collagen and proteoglycan are close and significant in the formation and functioning of connective tissue. Interactions occurring *in vitro* (Wood, 1960; Öbrink, 1975; Toole & Lowther, 1968) indicated that fibril formation could be profoundly affected by glycosaminoglycans. Whole-tissue analyses (Hoffman *et al.*, 1957) tentatively suggested a special relationship between coarse collagen fibres and dermatan sulphate.

Proofs of relationships *in vivo* have depended on ultrastructural methods. Early attempts to demonstrate tissue proteoglycan by electron microscopy (Smith *et al.*, 1967) demonstrated an orderly localization of proteoglycan with respect to collagen fibres in cornea and extracts of bovine nasal-septal cartilage. Others (Behnke & Zelander, 1970; Ruggieri *et al.*, 1975; Myers, 1976; Scott, 1980) confirmed a regular pattern of proteoglycans associated with collagen in intact connective tissue. In general it was not possible to locate the proteoglycan with respect to the ultrastructure of collagen fibres, as demonstrated by the banding pattern in the electron microscope. Smith *et al.* (1967) interpreted punctate staining by acid (pH 1.5) bismuth nitrate solutions as evidence for binding of cartilage proteoglycan (presumably chondroitin 4-sulphate-rich) to collagen fibres at the a and b bands. However, contrast was poor, and it is possible that artifacts occurred, as a consequence of the acid staining milieu. We now present results obtained with new reagents (Scott, 1980) in a more specific ('critical-electrolyte-concentration') method, at close-to-neutral pH, which, by reason of higher contrast than that available previously, enable the proteoglycans to be localized unambiguously with respect to the collagen banding pattern.

Materials and methods

Preparation of tissue samples

Tendon fibres were teased in iso-osmotic saline (0.9% NaCl) from the tails of mature (70-day-old) and young (2-, 7- and 25-day-old) rats. Short pieces were stained overnight in 2ml of aq. 0.05% Cupromeronic Blue [a cationic copper phthalocyanin dye used in a critical-electrolyte-concentration method to stain sulphated polysaccharides; dye (a) in Scott (1980)] containing 2.5% (v/v) glutaraldehyde, 0.1M- or 0.3M-$MgCl_2$ and 25mM-sodium acetate buffer, pH 5.7.

The tissue was rinsed three times, each for 5min, in a similar solution but without the dye. It was stained 'en bloc' in aq. 0.5% sodium tungstate for a total of 15min and dehydrated in an ethanol series, 0.5% sodium tungstate being included in the first [50% (v/v) ethanol] mixture (Scott *et al.*, 1981).

The tissue was embedded in Taab epoxy resin. Sections on copper grids were stained in 1% (w/v) phosphotungstic acid in 50% (v/v) ethanol (pH 3.5), rinsed for 30s in 1% (v/v) acetic acid (to maintain the low pH of about 3.5; Scott, 1971), stained in aq.

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0.5% uranyl acetate (pH 4.5) for 20 min and rinsed again in acetic acid for 30 s. Alternatively, grids were stained for 20 min in 0.5% uranyl acetate and rinsed for 30 s in distilled water.

Grids were examined in a Philips 301 electron microscope.

Hyaluronidase digestion

Single fibres from 2- and 70-day-old rats were digested with sheep testicular hyaluronidase (350 W.H.O. units/mg; BDH Chemicals) at 150 μg/ml in 0.05% sodium acetate solution containing 0.5 M-NaCl at room temperature overnight in the presence of proteinase inhibitors (see, e.g., Quintarelli *et al.*, 1978). Staining, embedding and sectioning were as described above.

Location of the proteoglycan filaments with respect to collagen banding

Collagen fibrils are often packed closely, particularly in mature tissue, with consequent uncertainty in 'assigning' proteoglycan filaments to the right fibre. When (*a*) the fibril was clearly separated from adjacent fibrils, or (*b*) the proteoglycan filament transversed the major part of a fibril, or (*c*) fibrils were very closely packed, but the collagen bands were in perfect register, the relationship of proteoglycan to the collagen fibril was unambiguous.

Results

Longitudinal sections of collagen fibrils stained with phosphotungstic acid and/or uranyl acetate showed a banding pattern that compared directly with that demonstrated for type 1 collagen (see, e.g., Chapman, 1974). Five deeply stained bands, a, b, c, d and e, were identified (Plate 1).

Proteoglycans appeared as electron-dense filaments orthogonally arrayed along the collagen fibrils (Scott, 1980). At least three different types of proteoglycan filament can be distinguished on the bases of their appearance and distribution: (1) broad, relatively long, densely stained rods, which do not appear to have any constant relationship to the fibrils; (2) finer filaments, of variable length, without apparent constant relationship to the fibrils; (3) filaments of similar width to (2), but with a constant relationship to the fibril, either in traversing the fibril at right-angles to its axis (the 'horizontal' filaments comprising the majority) or in joining two or more of the horizontal elements at right-angles, i.e. along the axis of the fibril.

We are concerned with the position of the horizontal elements of group 3 with respect to the banding pattern of collagen fibrils positively stained with phosphotungstic acid and UO_2^{2+}.

The course of proteoglycan filaments across collagen fibrils is not always absolutely straight and in perfect conformity with the collagen band, but the deviations are not great. These small variations are responsible for some, possibly most, of the uncertainty in localization.

From several hundred observations of proteoglycan filaments traversing collagen fibrils, 47 were unambiguously placed with respect to the collagen bands. Either (i) the bands were all individually identified by their characteristics, e.g. the 'triple' nature of band a (Plate 1), or (ii) the direction a→e of the fibril was established at several points along it, and one or more of the bands in the relevant D period were identified by their characteristic pattern. It was sometimes helpful to match, side by side, a less-clear pattern with one from a clearly stained and assigned fibril cut out of a picture at the same magnification. In every case the proteoglycan filament was almost equidistant from heavily stained zones (a + b), so that there was no doubt that it was in the c–e region, even when the collagen banding pattern was diffuse.

The proteoglycan filaments were most commonly (28 out of 47 observations) associated with the d band. Occasionally they occurred adjacent to the e band (six out of 47 observations) or between the two (13 out of 47 observations).

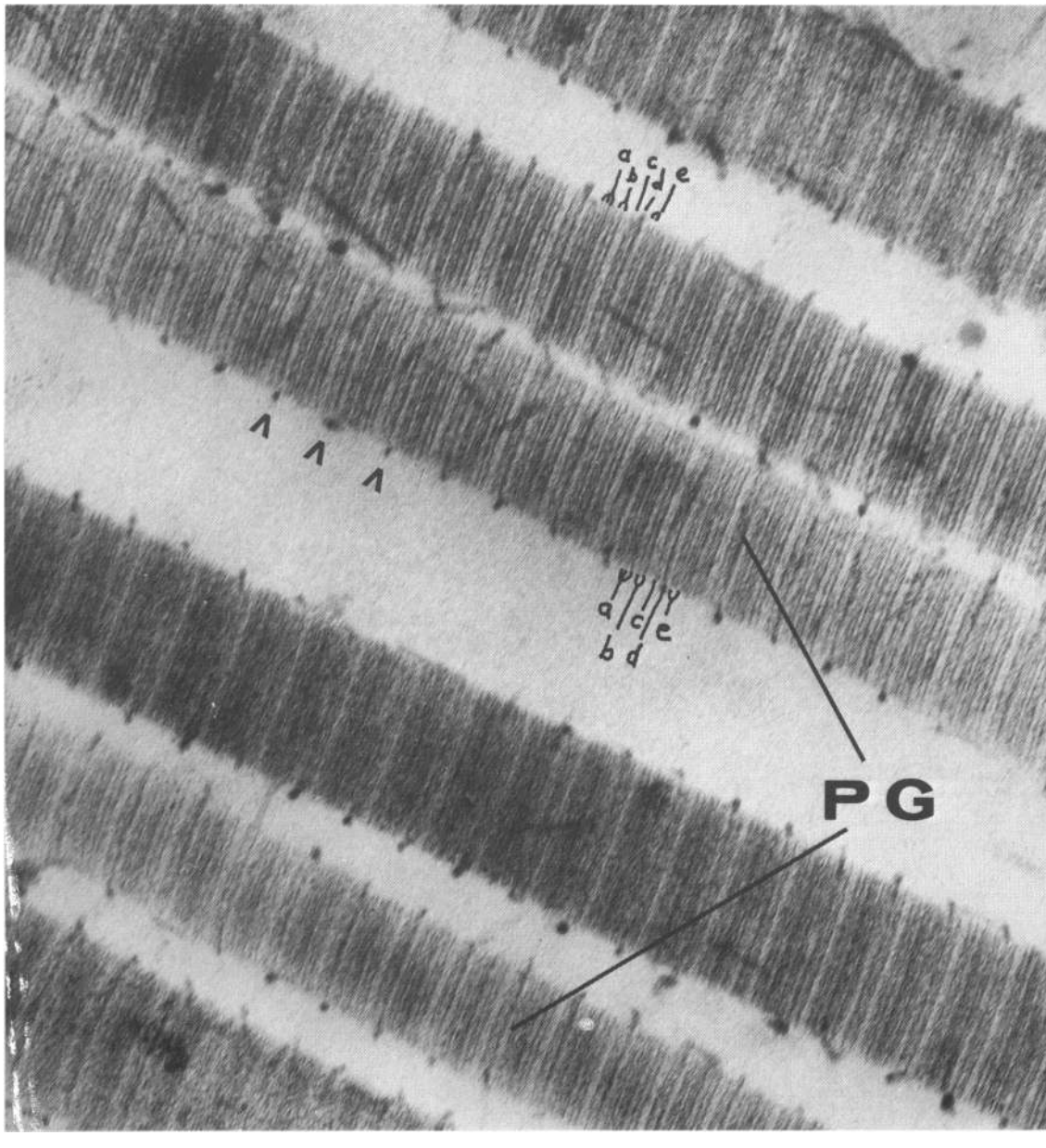
Both mature and younger tendons showed the same pattern, stained in the presence of either 0.3 M- or 0.1 M-$MgCl_2$.

Hyaluronidase digestion of 2-day-*post-partum* tissue removed almost all the proteoglycan observed in control tissues that had been incubated in buffer/proteinase-inhibitor solution, but without enzyme. Removal of proteoglycan from 70-day-*post-partum* tendon was patchy, and occurred to the same extent in control and experimental tissues.

Discussion

All ultrastructural stains for proteoglycan currently in use (Alcian Blue, Ruthenium Red, La^{3+}, Bi^{3+}, Cinchomeronic Blue etc.) precipitate the polyanion from an expanded solution-filling state to an almost 'dry' collapsed form (Scott, 1974, 1976). In terms of molecular structures, this artifact poses the questions (*a*) how specific to the proteoglycan is the precipitation reaction and (*b*) how far is the molecular morphology of the proteoglycan preserved in the precipitate? Further, since precipitates differing significantly in density from the surrounding medium must come to rest on some surface or fibril, it is necessary to relate the distribution of the artefact to that of the polyanion *in vivo*, e.g. by fixation of the tissue.

Cupromeronic Blue [dye (a) in Scott (1980)] stains all polyanions in the absence of salt, but added electrolyte prevents formation of dye–polyanion



EXPLANATION OF PLATE 1

Longitudinal section of 25-day-post-partum rat tail tendon stained en bloc with Cupromeronic Blue

The dye was 0.05% in 25 mM-sodium acetate buffer, pH 5.7, containing 0.1 M-$MgCl_2$ and 2.5% (v/v) glutaraldehyde; 0.5% sodium tungstate was included in the post-staining rinses with water and aq. 50% (v/v) ethanol. Sections in Taab epoxy resin were stained with 0.5% uranyl acetate to demonstrate the collagen banding pattern labelled a–e. Cross-sections of proteoglycan filaments are arrowed (^). Partial or complete perifibrillar hoops of proteoglycan are often visible (PG). Magnification ×177000.

complexes at concentrations of electrolyte (the critical electrolyte concentrations) that depend on whether the anionic group of the polyanion is sulphate, carboxy or phosphate. At 0.3 M-$MgCl_2$, the uptake of dye is relatively specific for sulphate-containing polyanions, polycarboxylates and poly-(ester phosphates) remaining unstained (Scott, 1980).

Because it is tetracationic, it can combine simultaneously with the polyanion and solution anions, e.g. tungstate (Scott, 1980). Binding of tungstate to the polyanion–dye complex results in a considerable increase in electron density, permitting the easy observation of stained proteoglycan even against the intensely stained background of phosphotungstic acid- and UO_2^{2+}-treated collagen fibres. Tungstate alone stains only the collagen fibre, faintly and diffusely, with no sign of proteoglycan filaments.

The evidence for specific staining of proteoglycans from the characteristic behaviour of Cupromeronic Blue in the critical-electrolyte-concentration system is corroborated by the hyaluronidase-digestion experiment, which resulted in the loss (from 2-day-*post-partum* tendon) of most of the stained orthogonal array. This was due to glycanase action on polysaccharide side chains, rather than proteolysis of the proteoglycan polypeptide core by proteinases in the tissue or the hyaluronidase preparation, since EDTA, benzamidine and soya-bean trypsin inhibitor were present during digestion. The more complete disappearance from 2-day-*post-partum* tendon, as compared with 70-day-*post-partum* tendon, may be due in part to easier access of enzyme through the tissue to the substrate; the former tendon contains only about 35% of the collagen content per g dry wt. of that of the older tendon (Scott *et al.*, 1981). In addition, there is a change in relative proportions of chondroitin sulphate to dermatan sulphate, which is by far the dominant glycosaminoglycuronan at 70 days *post partum* (Scott *et al.*, 1981). Dermatan sulphate is a co-polymer (Fransson & Havsmark, 1970) of galactosaminylglucuronic acid and galactosaminyl-iduronic acid. Since only the former unit is hydrolysed by testicular hyaluronidase, the less complete digestion of 70-day-*post-partum* proteoglycan may reflect the dominance of the latter.

Position of proteoglycan at the d band of collagen

Because the width of the proteoglycan filament varies slightly, it is true to say that it is centred on the d band, with some deviations towards the e band. This conclusion applies equally to tendons from 7-, 25- or 70-day-old rat tails, although the repeat banding distance (D) was found to increase considerably, from 53 to 63 nm, as the rat matured (Scott *et al.*, 1981).

Previous publications (Smith *et al.*, 1967; Doyle *et al.*, 1975; Myers, 1976) suggested that the a band was the site of proteoglycan association with collagen.

The apparent disagreement may be due to one or more reasons.

(i) The tissues were not the same. Smith and co-workers (Smith *et al.*, 1967; Smith & Frame, 1969) used cartilage and cornea, Myers (1976) synovium and ear cartilage. The cartilage collagen was likely to be type II and the proteoglycans were probably chondroitin sulphate-rich, compared with type I collagen (J. E. Scott, R. Timpl & K. von der Mark, unpublished work) and dermatan sulphate-rich proteoglycan (Scott *et al.*, 1981) in rat tail tendon.

(ii) The methodology may have influenced the outcome. The acid milieu (pH 1.5) before and during staining with bismuth nitrate (Smith *et al.*, 1967) would have converted tissue proteins, including collagen itself, into polycations. The sulphate ester groups of proteoglycan side chains would still be ionized (as confirmed by their ability to bind Bi^{3+}), and hence anionic proteoglycan could interact with polycationic protein. It is perhaps not surprising that proteoglycans were associated with the a band of collagen, which is the most cationic region (Doyle *et al.*, 1975). Polycation–polyanion interactions of this kind would not be expected in our system, which is at a higher pH, containing concentrations of electrolyte sufficient to suppress most coacervations etc. (Scott *et al.*, 1968).

In our experiments it is possible that glutaraldehyde fixed proteoglycan to a region with which it is not usually associated. This seems unlikely. It is necessary to postulate that only the d region has the capacity to fix proteoglycan via glutaraldehyde, although amino groups (lysine) are known to be present in other parts of the collagen structure (e.g. the a bands) to which glutaraldehyde should bind. Furthermore, it is implied that the proteoglycan moves uniformly, despite the presence of the precipitating dye, Cupromeronic Blue, to the region of the d band. Finally, we have observed regular orthogonal arrays of proteoglycan in the absence of glutaraldehyde, salt and sodium tungstate. Contrast and definition were inadequate to permit accurate localization with regard to the fibril structure.

Ruthenium Red has been used in conjunction with glutaraldehyde fixation, with somewhat contradictory results. Myers (1976) claimed localization at the a band, although the evidence is not clear, whereas Nakao & Bushey (1972) concluded that the d band was the binding site, and Doyle *et al.* (1975) interpreted Fig. 6 of Torp *et al.* (1975) as showing association 'between the a and d bands'.

Ruthenium Red is less specific as a proteoglycan stain (see, e.g., Scott, 1980) compared with Cupro-

meronic Blue in the critical-electrolyte-concentration system, and in general the published data are inconclusive.

Functional significance of localization at the d band

The d band in the gap zone is not cationic, which suggests that association is via short-range non-electrostatic forces. The cationic a and c bands (Doyle *et al.*, 1975) are roughly equidistant to each side. These may be the sites to which proteoglycan side chains extend and bind.

Two important functions probably occur at the 'gap zone': cross-linking of collagen fibrils, and calcification of collagenous matrices. Both functions would be expected to be influenced by the presence of a proteoglycan molecule with high charge and considerable excluded volume. Cross-links derive from the *C*-terminal and *N*-terminal telopeptides, which possess lysine residues oxidizable by lysyl oxidase to aldehydes, which then react with other lysine residues to form inter-molecular cross links (Bailey, 1975). Radial growth of the fibril, by accretion of collagen molecules or fusion of fibrils, could be hindered by restricted access to the cross-linking sites, due to proximity of the proteoglycan. It appears from electron microscopy that proteoglycan must be displaced during fibril fusion (Torp *et al.*, 1975; Scott *et al.*, 1981).

The first appearance of hydroxyapatite in newly calcified bone occurs in the holes of the gap zone (Fitton-Jackson, 1956). Tendon calcifies similarly (Nylen *et al.*, 1960). Dermatan sulphate-rich proteoglycan in the space later to be occupied by hydroxyapatite would probably have to be displaced or removed for calcification to occur. This would suggest that dermatan sulphate contents should be much lower in calcified as compared with uncalcified matrix, in line with early suggestions (Glimcher, 1959) that disappearance of 'muco-protein' was a necessary preliminary to the process of calcification. Although analyses of matrix before calcification to bone are not available, dermatan sulphate is present in fully calcified bone, but in very small amount (Scott *et al.*, 1979) (0.1 mg/g) compared with 1–2 mg/g of uncalcified young tendon (Scott *et al.*, 1981).

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References

Bailey, A. J. (1975) *Proc. Symp. Colston Res. Soc.* **26**, 115–127

Behnke, O. & Zelander, T. (1970) *J. Ultrastruct. Res.* **31**, 424–438

Chapman, J. A. (1974) *Connect. Tissue Res.* **2**, 137–150

Doyle, B. B., Hukins, D. L., Hulmes, D. J. S., Miller, A. & Woodhead-Galloway, J. (1975) *J. Mol. Biol.* **91**, 79–100

Fitton-Jackson, S. (1956) *Proc. R. Soc. London Ser. B* **146**, 270–281

Fransson, L.-Å. & Havsmark, B. (1970) *J. Biol. Chem.* **245**, 4770–4783

Glimcher, M. J. (1959) *Rev. Mod. Phys.* **31**, 359–393

Hoffman, P., Linker, A. & Meyer, K. (1957) *Arch. Biochem. Biophys.* **69**, 435–440

Myers, D. B. (1976) *Histochem. J.* **8**, 191–199

Nakao, K. & Bushey, R. I. (1972) *Exp. Mol. Pathol.* **17**, 6–13

Nylen, M. U., Scott, D. B. & Mosley, V. M. (1960) *Publ. Am. Assoc. Adv. Sci.* **64**, 129–142

Öbrink, B. (1975) *Proc. Symp. Colston Res. Soc.* **26**, 81–92

Quintarelli, C., Vocaturo, A., Rodén, L., Bellocci, L. & Vassallo, L. M. (1978) *Connect. Tissue Res.* **5**, 237–248

Ruggieri, A., Dell Orbo, C. & Quacci, D. (1975) *Histochem. J.* **7**, 187–197

Scott, J. E. (1971) *J. Histochem. Cytochem.* **19**, 689–691

Scott, J. E. (1974) in *Normal and Osteoarthritic Articular Cartilage* (Ali, S. Y., Elwes, M. W. & Leaback, D. H., eds.), pp. 19–32, Institute of Orthopaedics, London

Scott, J. E. (1976) *Ann. Méd. Reims* **13**, 63–67

Scott, J. E. (1980) *Biochem. J.* **187**, 887–891

Scott, J. E., Dorling, J. & Stockwell, R. (1968) *J. Histochem. Cytochem.* **16**, 383–386

Scott, J. E., Kyffin, T. W. & Hughes, E. (1979) in *Glycoconjugates* (Schaur, R., Boer, P., Buddecke, E., Kramer, M. F., Vliengenhardt, J. F. G. & Wiegandt, H., eds.), pp. 566–567, G. Thieme, Stuttgart

Scott, J. E., Orford, C. R. & Hughes, E. M. (1981) *Biochem. J.* **195**, 573–581

Smith, J. W. & Frame, J. (1969) *J. Cell Sci.* **4**, 421–436

Smith, J. W., Peters, T. J. & Serafini-Fracassini, A. (1967) *J. Cell Sci.* **2**, 129–136

Toole, B. P. & Lowther, D. A. (1968) *Biochem. J.* **109**, 857–866

Torp, S., Baer, E. & Friedman, B. (1975) *Proc. Symp. Colston Res. Soc.* **26**, 223–250

Wood, G. C. (1960) *Biochem. J.* **75**, 605–612