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Collagen–Proteoglycan Interactions

LOCALIZATION OF PROTEOGLYCANS IN TENDON BY ELECTRON MICROSCOPY

John E. SCOTT
Department of Medical Biochemistry, University of Manchester Medical School, Oxford Road, Manchester M13 9PT, U.K.

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Proteoglycan in foetal- and adult-rat tail tendon and adult-rabbit achilles tendon was stained for electron microscopy with a cationic phthalocyanin-like dye, based on cinchomeronic acid, in a 'critical electrolyte concentration' method [Scott (1973) *Biochem. Soc. Trans.* **1**, 787–806). Provided that the tissue was fixed with glutaraldehyde or formaldehyde, regular orthogonal perifibrillar arrays of filamentous material (proteoglycan) were observed, but no intra-fibrillar proteoglycan was seen. Specific proteoglycan–collagen interactions are inferred, and a model is proposed. Without fixation, the filamentous arrays disaggregated in the $MgCl_2$ solutions (0.3 M) used during staining. End-to-end proteoglycan aggregation is implied. Tendon and cartilage are compared. Problems of electron-histochemical localization of extended space-filling polyanions by the use of cationic electron-dense precipitants are discussed, particularly polyanion-domain collapse, specificity of staining and fixation. A two-stage staining procedure that markedly enhances contrast is described, based on the multivalent nature of the dye, and the consequent anion-exchange properties of the dye–polyanion complex.

Studies *in vitro*, particularly in solution (e.g. Öbrink, 1973), have demonstrated that proteoglycan or single glycosaminoglycan chains can interact with collagen, with some specificity. In a tissue, however, if a given proteoglycan was distant from a collagen fibre, one would not expect specific interactions between them. The answer to the primary question, are the proteoglycans so placed that they can interact with the fibres, requires an ultrastructural study of the tissue, e.g. by electron microscopy.

The electron density of proteoglycans is similar to that of the surrounding proteins, and the contrast must be increased by positive or negative staining if they are to be seen in the electron microscope. In tissues it is easier to enhance the electron density of polyanions by positive staining. The problems of the electron-histochemistry of proteoglycans (and other water-soluble high-molecular-weight polyanions) are quite different from those posed by condensed structures, such as fibrils, membranes and organelles.

Three aspects are discussed in the present paper: (1) specificity of the reagent with which to detect the proteoglycan; (2) the 'collapse' of the expanded polyanion in the process of staining, which can produce gross artifacts; (3) fixation of the proteoglycan, so that during staining etc. the proteoglycans do not move with respect to static components, such as cells and fibres.

The relevance of the findings to collagen–proteoglycan interactions are discussed. Preliminary accounts of some of this work have appeared (Scott *et al.*, 1977; Scott, 1979).

Materials and Methods

Materials

Adult-rat tail tendons were teased with needles in iso-osmotic saline (0.9% NaCl) to the smallest fibres that could be conveniently handled. Tails from 19-day foetal rats were used entire.

Rabbit achilles tendon was treated as described for the rat tail.

Two copper-containing tetra-azaporphin dyestuffs were synthesized (Scott, 1972). One, based on cinchomeronic acid (Fig. 1*a*), used in most experiments is referred to below as cinchomeronic dye, instead of the semi-systematic name of *N'N''N'''N''''*-tetramethyltetra-azaporphin tetrakis-

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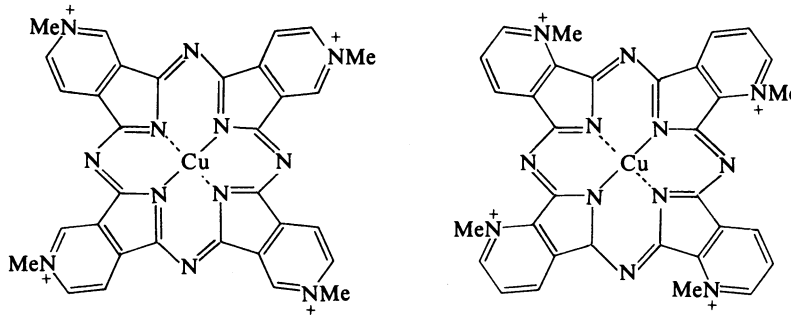


Fig. 1. *Structure of (a) cinchomeronic dye and (b) quinolinic dye*

methanosulphate. The second dye, based on quinolinic acid (Fig. 1*b*) is referred to below as quinolinic dye. This dye was prepared in its palladium and platinum as well as copper forms, by essentially similar methods. Araldite resin and other embedding materials were from Taab Laboratories, Reading, Berks., U.K.

Sodium tungstate, phosphotungstic acid and uranyl acetate were AnalaR grade from BDH Chemicals, Poole, Dorset, U.K. All other chemicals were of the best laboratory grade obtainable.

Methods

Tendons were stained overnight at room temperature (21°C) in cinchomeronic or quinolinic dye at 0.05% in 25 mM-sodium acetate buffer, pH 5.6, containing $MgCl_2$ at 0.1, 0.3 or 0.5 M. Formalin (2.5%, w/v) or glutaraldehyde (2.5%, w/v) was included where indicated. The tissues were twice rinsed in buffer containing $MgCl_2$ at the same concentration as in the staining solution, for about 3 min altogether. The tissue was washed in distilled water, taken through graded water/ethanol mixtures to 100% ethanol, and then through epoxypropane/ethanol mixtures to pure epoxypropane. Embedding in Araldite was by standard procedures (e.g. Geyer, 1977).

Staining with 0.5% sodium tungstate in 50% (v/v) ethanol/water mixtures was performed on thin sections of Araldite on a Formvar-coated grid for 20 min at room temperature. The sections were washed briefly in distilled water and dried in air.

Phosphotungstic acid or uranyl acetate staining was performed in accordance with standard procedures (e.g. Geyer, 1977).

Sections were examined either in a Philips 800 or an AEI 6B electron microscope.

Results

Mature-rat tail tendon

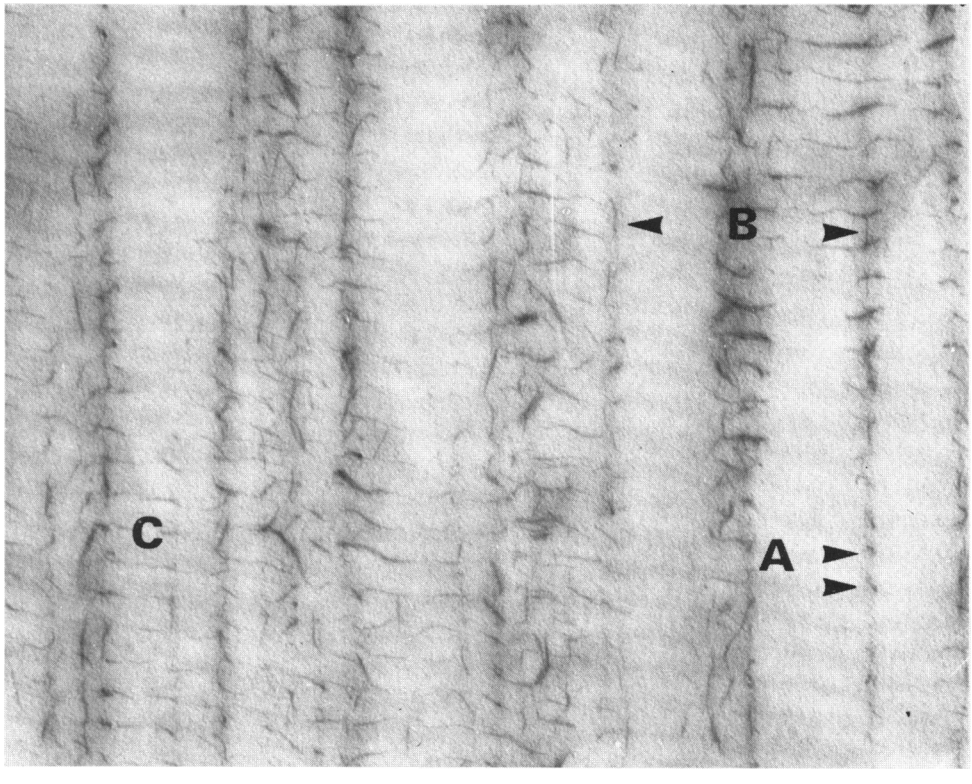
This was examined in longitudinal section. (Foetal-rat tail tendon could often be observed longitudinally and transversely in the same section.)

Tissue stained in cinchomeronic dye solutions containing glutaraldehyde or formaldehyde showed clearly delineated orthogonal arrays of filamentous structures against an almost unstained background in which the outline of collagen fibres was discernible (Plate 1). The contrast between proteoglycan filaments and collagen fibres was markedly increased by the sodium tungstate treatment of dyed tissue. Control tissues, which were immersed in buffer/salt/glutaraldehyde solution without dye, showed no trace of the electron-dense filamentous array. This was true whether the dyed section was exposed to sodium tungstate solution subsequent to embedding in Araldite or not. Material stained with sodium tungstate showed banding characteristic of collagen, but only faintly in comparison with that produced by staining with phosphotungstic acid or UO_2^{2+}.

Proteoglycan was present outside the fibrils.

The horizontal proteoglycan filaments were in the region between the tungstate-stained collagen bands. The vertical components of the orthogonal array seemed to be 'stepped' about the fibre. In general, vertical filaments did not run into other vertical filaments, but had contacts with horizontal filaments (Plate 1). 'Horizontal' and 'vertical' are defined with reference to the collagen 67 nm bands, which are 'horizontal'.

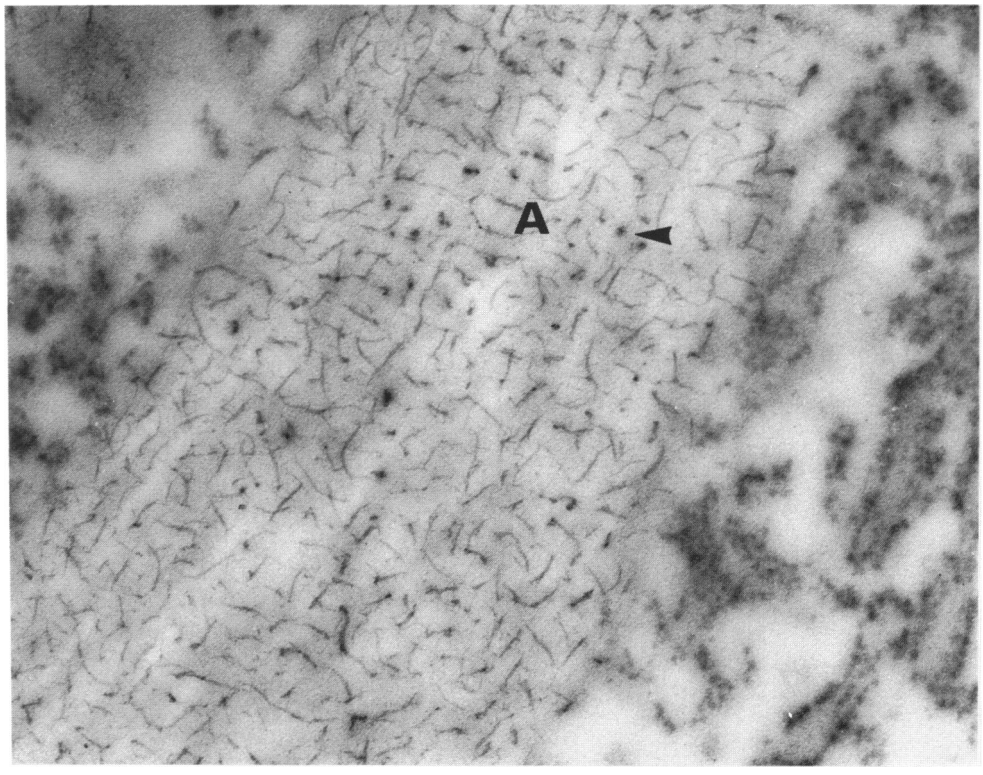
Tissues stained in solution with buffer containing 0.3 M-$MgCl_2$ but no glutaraldehyde showed a completely different picture (Plate 4). The orthogonal array was no longer visible. Only dispersed filaments were observed, of similar thickness to the filaments in fixed tissue. Their apparent lengths varied, but most were about 70 nm long. Tissue treated overnight with buffer containing 0.3 M-$MgCl_2$ but without dye or glutaraldehyde and stained with phosphotungstic acid in Araldite sections showed considerable disorganization of the fibrils, in which



EXPLANATION OF PLATE 1

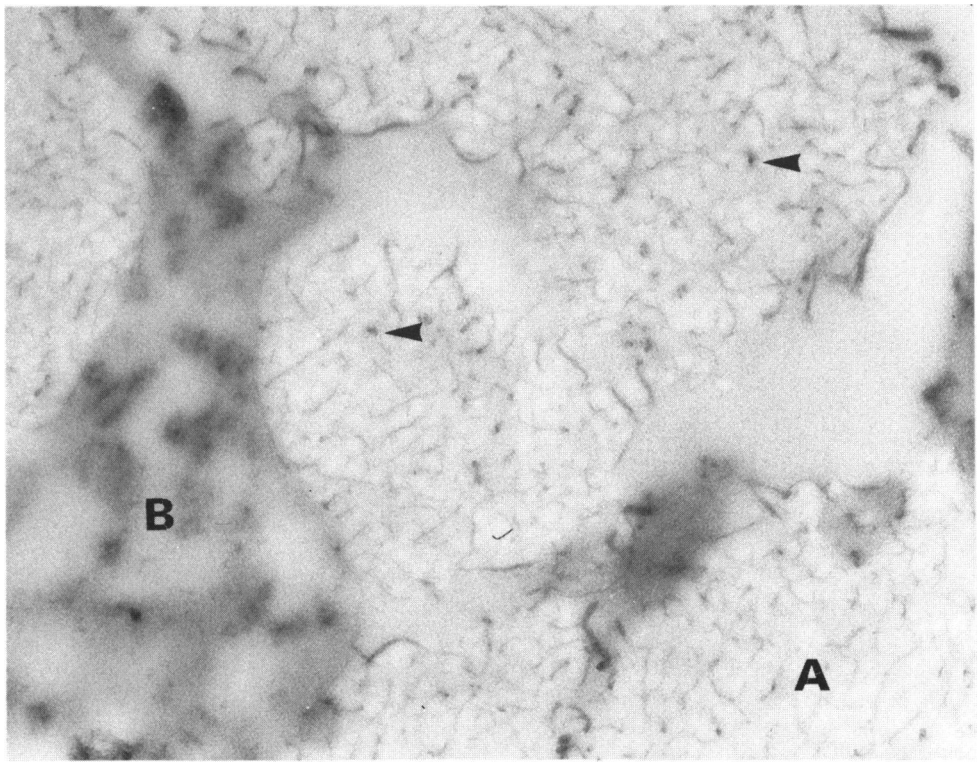
Adult-rat tail tendon, longitudinal section, stained with cinchomeronic dye (0.05%) in 0.3 M-$MgCl_2$/*25 mM-sodium acetate buffer, pH 5.6, containing 2.5% glutaraldehyde (16 h) and embedded in Araldite as described in the text.*

The thin Araldite section was stained in 0.5% sodium tungstate in aq. 50% (v/v) ethanol for 20 min. Where the fibril is cut on the diameter, the perifibrillar filaments of proteoglycan are seen in cross-section, e.g. A. A tangential cut leaves almost the complete hoop of proteoglycan filament, e.g. in the vicinity of C. The 'vertical' components of the orthogonal array are visible, e.g. at B. Magnification × 85 000.



EXPLANATION OF PLATE 2

Foetal (19-day) rat tail, fixed overnight in 2.5% glutaraldehyde at room temperature, stained in cinchomeronic dye (0.05%)/0.1 M*-$MgCl_2$/25 m*M*-sodium acetate buffer, pH 5.6, (16 h) and embedded in Araldite as described in the text*
The thin Araldite section was stained in 0.5% sodium tungstate in aq. 50% (v/v) ethanol for 20 min. Bundles of fine fibrils are seen in longitudinal section at A, in contact with cellular material. Longitudinal and horizontal components of the proteoglycan orthogonal arrays are visible throughout, as are cross-sections of proteoglycan filaments (arrowed). Magnification × 85 000.



EXPLANATION OF PLATE 3

Foetal-rat tail, showing transverse sections of collagen fibrils (A) in contact with cellular material (B)
Staining was as described for Plate 2. Transverse proteoglycan filaments can be seen interwoven between the fine collagen fibrils, which show as light, almost circular, areas 20–30nm in diameter. Cross-sections of axial proteoglycan filaments are also visible (arrowed). Magnification × 85000.

EXPLANATION OF PLATE 4

Adult-rat tail tendon stained in cinchomeronic dye (0.05%)/0.3 M-$MgCl_2$/*25* mM-*sodium acetate buffer, pH 5.6 (16 h), without fixation, and embedded in Araldite as described in the text*

Dispersed elements of the proteoglycan array are seen (A) against a faint background of collagen fibres. The proteoglycan units are typically 60–70 nm in length.

the complete characteristic collagen pattern was only intermittently visible.

More-polar embedding media, e.g. hydroxyethyl methacrylate, were sometimes used instead of Araldite. The pictures were similar to those from Araldite-embedded tissue, but less clear, and the contrast was less.

Results obtained with the quinolinic dye (Fig. 1*b*) in buffer/salt/fixative solutions followed by Araldite embedding were very similar to those obtained with the cinchomeronic dye.

Adult-rabbit achilles tendon showed orthogonal arrays similar to those of rat tail tendon.

Foetal-rat tail

Not only were longitudinal and transverse sections of collagen bundles observed in the same thin section, but cartilage, cells and mast cells were also often visible.

The fibres were very thin, but the orthogonal array of proteoglycans was as clearly visible as in the adult. The horizontal filaments were separated by about the same distance as that between the collagen bands. No proteoglycan was visible in the interior of the fibres.

Transverse sections showed proteoglycan fibres interwoven between the collagen fibrils (Plate 3).

Collagen fibres in foetal-rat tail transverse sections showed as very light areas, compared with the proteoglycan (Plate 3). After staining with phosphotungstic acid, they were dark, proving that they were protein fibres, and not spaces.

Cartilage in foetal-rat tails stained with the copper, palladium or platinum forms of quinolinic dye in 50mM-$MgCl_2$ under identical conditions showed increasingly electron-dense proteoglycans in the order Cu < Pd < Pt.

With Pb^{2+} or UO_2^{2+} no structures of the kind detected by cinchomeronic dye were present, whether the metal was used on Araldite or hydroxyethyl methacrylate sections.

Discussion

Previous attempts to localize proteoglycans in cartilage and other tissues failed on one or more of three counts. First, the staining reagent was not adequately specific, or it was used in conditions in which non-specific staining was possible. Secondly, the affect of the reagent on the morphology of the proteoglycan was not recognized. Thirdly, the water-soluble proteoglycan was not 'anchored', so that the 'molecular collapse' brought about by the staining reaction (Scott, 1976), coupled with the mobility of a water-soluble molecule in aqueous media, could have caused gross translocation of the proteoglycan.

Reagent specificity

The validation of cinchomeronic dye as a reagent for proteoglycans is easy because staining is visible to the eye. Its specificity has been checked on isolated polyanions (Scott, 1972). This has not been done for UO_2^{2+}, Pb^{2+} or most other cationic electron-dense reagents. Cinchomeronic dye fits the pattern of cationic precipitants, e.g. cetylpyridinium, Alcian Blue etc., which are widely used in biochemistry and histochemistry (Scott, 1973). As a quaternary ammonium precipitant, it is displaced easily from polycarboxylates by $MgCl_2$, but from poly-ester-sulphates with greater difficulty, i.e. at higher $MgCl_2$ concentrations. Quinolinic dye behaves similarly, but it differs from cinchomeronic dye in having high affinity for nucleic acids, from which (particularly RNA) it is displaced by $MgCl_2$ only with difficulty (Scott, 1973). Cinchomeronic dye does not have high affinity for nucleic acids because of its inability to intercalate into stacked base-pairs. It is bound to tissue polyanions predominantly by electrostatic forces (Scott, 1972). In $MgCl_2$ concentrations above 0.3M, poly-ester-sulphates are the commonest polyanions in mammalian tissues that stain with cinchomeronic dye (Scott, 1972).

Attempts to improve the specificity, or to validate staining by Pb^{2+}, UO_2^{2+} etc. by using, e.g. hyaluronidase, to digest materials that would otherwise stain, have had limited success. Enzymatic removal of considerable amounts of large molecules probably allows parallel losses of components that were not digested by the enzyme. Even less acceptable is the use of proteolytic enzymes, on the grounds that the proteoglycan polypeptide core is split, and electron-dense material lost as a consequence must have been proteoglycan. The arguments against hyaluronidase etc. apply to proteolytic enzymes with greater force.

Polyanion collapse

As yet it is not possible to attach sufficient electron-dense material to an extended space-filling proteoglycan to show all the ramifications of molecular structure in tissues. With isolated proteoglycans this was done by concentrating the polyanion into a film against a uniform background (Rosenberg *et al.*, 1970). At the other extreme is the fully collapsed form of the proteoglycan, e.g. in the form of a La^{3+} salt. Although electron density is thereby much enhanced, the details of molecular morphology are lost.

Cinchomeronic dye brings about a partial collapse, and Alcian Blue, which is about four times the size of cinchomeronic dye (Scott, 1972), brings about an even less complete collapse, with consequently less contrast, and less detail of molecular morphology (Scott, 1976).

Cinchomeronic dye is the reagent of choice. It can be used in the critical-electrolyte-concentration system (Scott, 1973) to enable predominantly sulphated polyanions to be stained, and, in bringing about the collapse of proteoglycan from the water-swollen to the almost 'dry' form of the dye complex, the change is less drastic than in the case of, e.g., La^{3+} (Scott, 1976). The artifacts produced in the tissue show similarities to those of spread film preparations obtained on isolated proteoglycans (Scott, 1976).

Since neither Pb^{2+} nor UO_2^{2+} is an efficient precipitant of proteoglycans, they cannot be used in the same way as cinchomeronic dye. They are used on embedded material, and the collapse of the proteoglycan caused by the dehydration steps before plastic embedment brings about a more haphazard picture than that produced by a precipitation cation.

In this context, Ruthenium Red (Luft, 1965) is superior to Pb^{2+}, UO_2^{2+} and La^{3+}, but it is not easy to manipulate its specificity towards particular groups of polyanions (Gustavson, 1970; Yamada & Hoshino, 1972).

Enhancement of dye electron density

The electron density of the cinchomeronic dye-precipitated proteoglycans is sufficient for many applications, but not enough to see fine details of proteoglycan structure. The electron density of cinchomeronic dye was increased by replacing the copper atom with palladium or platinum (Scott *et al.*, 1977, and unpublished work). Low yields were obtained after the long syntheses, and the increase in electron density achieved (about 4-fold, on the basis of depth of image on photographic paper) was not an adequate return.

An alternative approach is available, based on the multivalent character of cinchomeronic dye. As shown in Fig. 2(*a*), the four charges on the dye can bind to four negative sites on a polyanion. However, diffusible anions in solution, e.g. Cl^- from $MgCl_2$, can replace the polymer-bound anions (Fig. 2*b*), and much more dye is then bound per unit of substrate. This effect was demonstrated with Alcian Blue (Scott *et al.*, 1964). The polyanion-dye-diffusible anion complex constitutes a particle of anion-exchanger, and Cl^- can be replaced by a different anion. If the replacing anion is more electron-dense than Cl^-, e.g. tungstate, WO_4^-, the electron density of the complex is greatly increased (see the Results section). In principle, three tungstate ions could bind to cinchomeronic dye in a polyanion complex.

Distribution of proteoglycan in tendon

The proteoglycan is distributed on the outside of the collagen fibres. No proteoglycan is visible within either the very fine foetal-rat tail fibres or the large mature fibrils in rat tails or rabbit tendons.

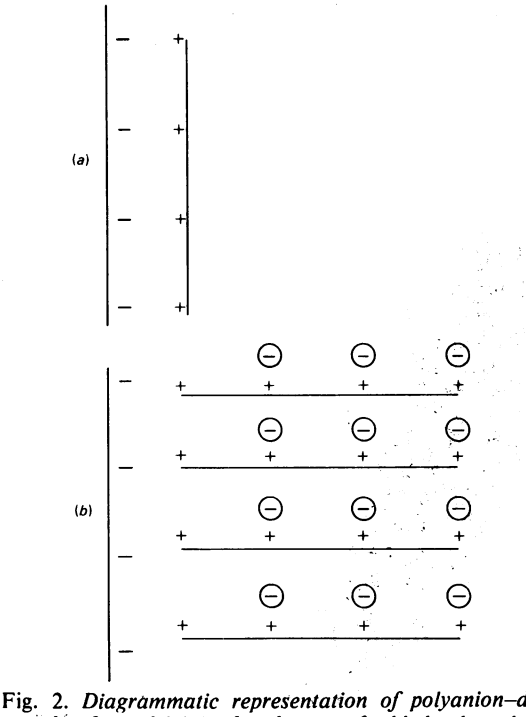


Fig. 2. *Diagrammatic representation of polyanion–dye complex formed (a) in the absence of added salt and (b) with added salt*

In (*a*), four positive charges on a quadrivalent dye, e.g. cinchomeronic dye, interact with four negative charges attached to a polymer. In (*b*), three of the four positive charges on the dye interact with diffusible anions ⊖ from, e.g., $MgCl_2$ in solution.

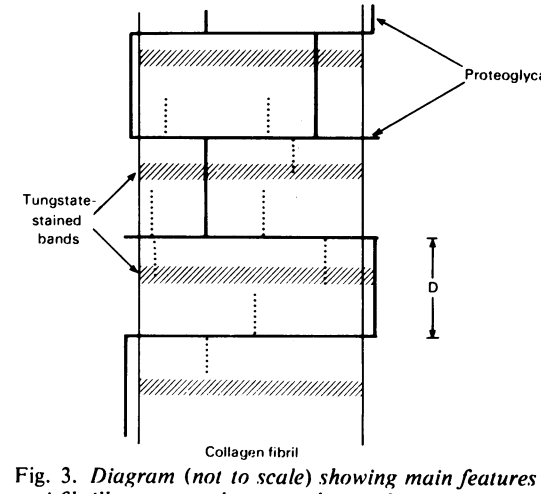


Fig. 3. *Diagram (not to scale) showing main features of peri-fibrillar proteoglycan orthogonal array in adult tendon, as inferred from cinchomeronic dye and sodium tungstate staining at pH 5.6 in 0.1 M- or 0.3 M-$MgCl_2$*

D indicates collagen banding distance (about 62 nm). Broken lines, glycosaminoglycuronan side chains; continuous lines, proteoglycan polypeptide cores.

The meshwork of proteoglycan filaments consists of vertical and horizontal components. The regularly spaced horizontal components are separated by the collagen banding repeat distance (about 65 nm). These results indicate that there is a specific interaction between proteoglycan and collagen. It is otherwise difficult to explain the very regular distribution of proteoglycan filaments along the collagen fibre.

After the use of tungstate, the collagen banding is clear although faint. The proteoglycan filaments are in the less-stained region between the tungstate-stained bands. Presumably tungstate binds to the cationic bands of the collagen fibre. Assuming analogies between tungstate and phosphotungstate staining, the proteoglycan filament is attached to the non-polar regions of the collagen fibre. This is unexpected, since the negatively charged proteoglycan must be attracted electrostatically to the cationic bands of the collagen fibril. These considerations can be reconciled in a model assuming that the proteoglycan consists of a polypeptide core with polysaccharide side chains distributed symmetrically, and with the polypeptide placed between the collagen cationic bands towards which the polysaccharide chains are directed (Fig. 3). The pattern observed would be of the proteoglycan with the carbohydrate side chains collapsed on to the polypeptide core by precipitation during staining, as is common in proteoglycan histochemistry (Scott, 1976). This model can accommodate a protein–protein interaction between the proteoglycan core and collagen.

In the absence of fixative, quite low concentrations of salt (0.3 M-$MgCl_2$) disaggregate the array of fibre-enclosing proteoglycans. The proteoglycan appears as single rod-like filaments of about the same length as the collagen banding distance (65 nm). This suggests (*a*) that the interactions holding the arrays in position are weak and (*b*) that the orthogonal meshwork is an end-to-end aggregate, a different type of aggregation from that observed with cartilage proteoglycans.

Whether hyaluronic acid is involved cannot be determined, since the $MgCl_2$ concentrations used in these experiments prevent the staining of hyaluronate.

Tendon proteoglycans contain considerable amounts of dermatan sulphate (Anderson, 1975), and experiments in solution demonstrated that dermatan sulphate interacts more strongly with collagen than does chondroitin 4-sulphate (Öbrink, 1973). These findings are relevant to my own from the electron microscopy of tendon, and to the fact that the picture in tendon is quite different from that observed in cartilage with the same reagents and methodology (Scott, 1976). The very fine collagen filaments in the cartilage territories are in a position to interact with large amounts of proteoglycan, which is the converse of the situation in mature tendon. It appears that in cartilage territories most of the collagen can interact with some of the proteoglycan, whereas in tendon most of the proteoglycan can interact with some of the collagen.

Note Added in Proof (Received 19 March 1980)

The dye based on quinolinic acid is now available from BDH Chemicals (Poole, Dorset, U.K.) under the name 'Cuprolinic blue'.

My thanks are due to Dr. T. W. Kyffin, Dr. C. J. P. Jones and Mrs. J. Webb for their help in making the dyes and obtaining the electron micrographs.

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