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The structure of interfibrillar proteoglycan bridges ('shape modules') in extracellular matrix of fibrous connective tissues and their stability in various chemical environments

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ABSTRACT

Collagen fibrils in extracellular matrices of connective tissues (tendon, cornea, etc.) are bridged and linked by the anionic glycosaminoglycans (AGAGs) of the small proteoglycans (decoron, etc.). It was proposed that these bridges and ties maintain the collagen fibril dispositions in relation to each other, helping to define tissue shape, and hence called shape modules. This investigation describes chemical and physicochemical conditions in which these structures are stable and what treatments cause their disruption. The effects on fixed and unfixed sections of tendon, cornea, lung and ear from rat, mouse and rabbit of pH, electrolyte concentration, EDTA, mercaptoethanol, hydrogen peroxide, free radicals, periodate, acetylation, urea, nonionic detergent and organic solvents were assessed by staining with Cupromeronic blue or Alcec blue in CEC techniques to localise AGAG bridges or their disintegration products. Ca^{2+} was not involved in the structures, oxidation/reduction had no effect and Triton X100, a nonionic detergent did not damage them. They were stable between pH 4.5 and 9.5. Periodate as a glycol-cleaving reagent did not affect them. High concentrations of urea (> 2.0 M) and $MgCl_2$ (0.5 M) disrupted the tissues. The combination of Triton and urea at concentrations too low to cause damage separately was disruptive. Free radicals in periodate solutions were damaging. Organic solvents caused collapse and rearrangements of the AGAG filaments. Acetylation caused considerable disruption of shape modules. Dermochondan but not keratan sulphate AGAGs were removed by treatment with NaOH. After fixing with glutaraldehyde only free radical and NaOH treatments were severely disruptive of shape modules. The results are compatible with a previously proposed structure for the shape modules, stabilised by hydrophobic and hydrogen bonding.

Key words: Collagen; chondroitin sulphate; decoron; Cupromeronic blue.

INTRODUCTION

The extracellular matrix (ECM) of connective tissue maintains and defines the shapes of tissues and organs. ECM is composed mainly of collagens and proteoglycans (PGs) of several different families. Soft fibrous tissues (tendon, skin, cornea, etc.) contain predominantly type I collagen associated with 'small' PGs such as proteodermatan sulphates, PDSs (e.g. decorin) and proteokeratan sulphates (PKSs), of molecular mass ~ 100 kDA, $\sim 50\%$ of which is protein, the rest being anionic glycosaminoglycan (AGAG) (Scott, 1995). When tissues are stained with the electron-dense reagent Cupromeronic blue (CB) under specific critical electrolyte concentration (CEC) conditions the AGAGs appear in electron micrographs as filaments, regularly and orthogonally bridging between and across collagen fibrils (Scott, 1992*a*), often spanning several at a time. The PGs bind via their protein cores at specific sites in the gap zone of the collagen fibrils (Scott, 1996).

The interfibrillar AGAG bridges were proposed to be an important part of tissue shape maintenance, organising collagen fibrils by tying them together. Bridge lengths vary from tissue to tissue. Tendon collagen fibrils are close together, hence the bridges

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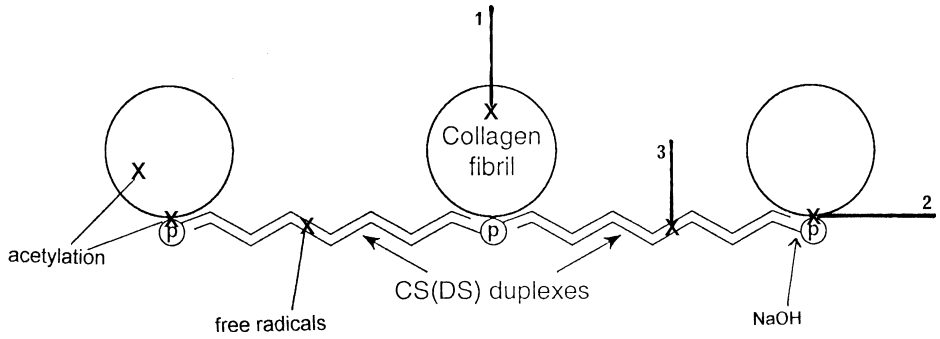

Fig. 1. The 'shape module' AGAG chains, usually either chondroitin or dermochondan sulphates (CS or DS), bridge and link between collagen fibrils, to which they are noncovalently attached via their protein carriers (P). In cornea similar structures contain the AGAG keratan sulphate in addition to CS/DS. The fibrils are thereby maintained in their orientations, helping to define ECM, and hence tissue, shape. It was proposed (Scott, 1992) that the bridges consist of antiparallel AGAG molecules of very similar lengths which may determine the separation of the collagen fibrils, being shorter where fibrils are close together (skin, sclera) and longer in corneal stroma where fibril separation is greater.

The 3 regions where the treatments used in this paper could lead to a change in stability of the bridges are 1, the collagen fibril itself, 2, the PG protein:collagen fibril interface and 3, the AGAG aggregate. Several treatments caused severe disruption of the shape modules in tissue: NaOH, organic solvents, free radicals and acetylation. NaOH chemically cleaved the CS(DS) chains, solvents caused a collapse of the bridges, free radicals fragmented the AGAG structures and acetylation probably affected both the integrity of the collagen fibrils and the PG:collagen interface. Acid (< 4.0) pH caused disruption of the tissue as a whole, although between pH 4.5 and pH 9.0 there was no apparent change in shape module structure.

are short. Corneal collagen fibrils are wider apart, requiring longer bridges to maintain the regular collagen fibril distribution, without which the cornea would not be transparent (Scott, 1995). Since the basic structure (Fig. 1), consisting of collagen fibril PG→protein→AGAG⇔AGAG←PG protein collagen fibril, is repeated a very large number of times in a given volume of ECM, they are regarded as modules, consequently termed the 'shape module'.

The first human pathology in which the shape modules were missing from matrices produced by skin fibroblasts, has been observed in an electively aborted fetus (Dyne et al. 1997). A decoron gene knockout mouse has been described, with severe manifestations in skin ultrastructure (Danielson et al. 1997).

Patterns of collagen fibrils with orthogonal PG bridges in fibrous ECMs are present in echinoderms (Erlinger et al. 1993), birds and mammals (Scott, 1988). PG:collagen interactions have thus long played a fundamental role in the structure and function of connective tissues, having been conserved throughout animal evolution.

It is therefore of fundamental interest to know how stable these structures are and what forces maintain them. They resisted severe tensile stresses exerted on tendon (Cribb & Scott, 1995). In particular, it is important to know how changes in the milieu interieur could weaken or disrupt them. With this in mind, we assessed the stability of interfibrillar bridges in a variety of fibrous connective tissues using different types of reagent. We looked for the persistence of these bridges was examined using 12 different kinds of reagent: (1) at pHs varying between 2.0 and 13 (0.1 M NaOH solution, see section 10 below); (2) in $MgCl_2$ solutions; (3) in EDTA solution, in which Ca^{2+}, Mg^{2+} and other polvalent cations are sequestered. Ca^{2+} is often implicated in intermolecular bonds between biopolymers in tissues; (4) after nitrous acid treatment to eliminate primary amino groups; (5) after acetylation of amino groups; (6) after treatment with hydrogen peroxide, which could oxidatively cleave –S–S–bonds and generate free radicals ($OH^{\bullet}$); (7) after treatment with mercaptoethanol which reductively cleaves –S–S–bonds; (8) after periodate treatment which cleaves glycol groups in the uronate residues of dermochondan sulphate (formerly dermatan sulphate), inter alia; periodate may also generate free radicals (Scott & Page-Thomas, 1976); (9) after exposure to 0.1 M NaOH solution; (10) in urea solutions; (11) in Triton X100, a nonionic detergent, solutions; and (12) after immersion in organic solvents, of various polarities. Experiments (6) and (8) were performed with and without n-propanol (4% v/v), a free radical scavenger.

All investigations were performed on 10 μm tissue sections. Glutaraldehyde-fixed and unfixed tissues were compared, as described below. Treated and control sections were stained to demonstrate PGs and collagen fibrils.

The data are directly relevant to techniques for demonstrating PG:collagen fibril interactions by electron microscopy.

An abstract of part of this work has appeared (Thomlinson & Scott, 1997).

MATERIALS AND METHODS

Tissues

The following tissues were studied: 6–7, 8, 10, 10–12, 12 and 16 wk rat tail tendon (RTT): 11 wk rat ear (RE); 11, 14 wk rat lung (RL); 16 wk mouse cornea (MC) and 14 wk rabbit cornea (RC). They were removed from the animal shortly after death. RE, RL, MC and RC were immediately frozen in isopentane cooled in liquid N_2. Rat tails were frozen on a shelf in a freezer at −80 °C. Frozen tissues were stored at −80 °C.

Sections (10 μm) of frozen tissues on gelatin coated slides (Haigh & Scott, 1986) were immediately treated in 1 of 12 ways, as outlined below.

Stains

Cupromeronic blue (CB) was from Seikagaku, Tokyo. Alcec blue (AB) was made by the method of Scott (1980). Both dyes are electron dense precipitants of PGs that scaffold PGs during precipitation, maintaining their shape and position in the tissue (Scott, 1985). CB is unstable in alkali, in which AB is stable.

Fixation, staining and embedding

Standard glutaraldehyde fixation (when carried out before staining) was for 30 min at room temperature (RT) in 2.5% glutaraldehyde/25 mM NaAc at pH 5.8, followed by 3 washes in 25 mM NaAc at pH 5.8.

Standard staining was in solutions containing 0.1 M $MgCl_2$; 25 mM NaAc pH 5.8; 2.5% glutaraldehyde and 0.05% w/v CB or AB, for 3 h at 37 °C or overnight at RT (see individual expts). The sections were washed in a solution identical to that in which the stain was dissolved, stained further with 0.034 M sodium tungstate (Na_2WO_4), which increases the electron density of the AB and CB complexes (Scott, 1985) and then dehydrated in ethanol:H_2O mixtures before embedding in Spurr resin.

Where possible staining solutions were modified to carry the conditions in the test solutions into the staining step (see individual treatments for details). Collagen fibrils in ultrathin sections were stained with uranyl acetate (UA, 2% w/v) in 70% ethanol:H_2O.

In experiments on the effect of pH (samples treated at pH 2.0, 4.5, 7.0, 9.5), ultrathin sections were stained with 1% w/v silicotungstic acid (STA) in 50% v/v ethanol/H_2O solution for 20 min either at pH 2.7 (natural pH) or pH 5.2 (adjusted with ammonium acetate). Collagen banding patterns were clearer after the low pH STA, PG filaments showed more clearly after the high pH STA.

Simultaneous collagen staining and enhancement of AB electron density using STA instead of sodium tungstate was achieved with 7.0% w/v STA at pH 2.2 or pH 5.0 (containing 0.034 M NaAc, adjusted with NaOH), after staining with AB, before dehydration and embedding (samples treated at pH 4.0, 5.5, 7.0, 8.5).

Ultrathin sections were examined in a Philips 400 or Philips 420 transmission electron microscope.

Effects of various environments on PG interfibrillar bridges in tissues

1. *pH*. 16 wk RTT sections were immersed in solutions of pH 2.0, 4.0, 4.5, 5.5, 7.0, 8.5 and 9.5 for 15 min at RT. All solutions contained 0.14 M NaCl. pH 2.0 was obtained with 3% v/v acetic acid; pH 4.0, 4.5 and 5.5 with 25 mM NaAc/acetic acid buffers; pH 7.0, 8.5 and 9.5 with 25 mM Tris/acetic acid buffers. AB staining solutions included 0.1 M $MgCl_2$, 2.5% glutaraldehyde and 0.05 % w/v AB, adjusted to the same pH as that used in treating the tissue sections. Stain solution containing 25 mM NaAc was adjusted to pH 4.0, 4.5 and 5.5 using 5% v/v acetic acid, and for pH 7.0, 8.5 and 9.5 using 0.1 M NaOH added to 25 mM Tris HCl buffer. Sections were immersed in AB solutions at 37 °C for 4 h.

For experiments at pH 13, see section 9 below.

2. *Salt solutions*. Sections were immersed for 1 h at RT in 0.1 M or 0.5 M $MgCl_2$ containing 25 mM NaAc, adjusted to pH 7.0 with NaOH. 1.0 M $MgCl_2$ was used at pH 6.1. They were then stained with CB for 3 h at 37 °C.

3. *EDTA*. 16 wk RTT sections were demineralised by immersion in 1 of 3 different EDTA solutions. (i) Aqueous 25 mM disodium EDTA at pH 5.8 (adjusted with NaOH) containing 0.16 M NaCl, 70 mM NaAc, 2.5% v/v glutaraldehyde for 3 h at 37 °C, i.e. 'demineralisation in the absence of dye'. They were then washed ×3 in 0.1 M NaAc pH 5.8 and stained in 0.16 M NaCl, 2.5% glutaraldehyde, 25 mM NaAc pH 5.8 and 0.05% w/v CB, for 3 h at 37 °C. 0.16 M NaCl in the stain solution ensures specific staining of sulphated PGs according to the CEC principle (Scott, 1985). Control solutions, adjusted to pH 5.8 with acetic acid, were identical, except that EDTA was absent. (ii) Aqueous 25 mM disodium EDTA at pH 5.8 (adjusted with NaOH) containing 0.16 M NaCl, 70 mM NaAc, 2.5% glutaraldehyde, and 0.05 v/v CB for 3 h at 37 °C—i.e. 'demineralisation in

the presence of the dye'. Control solutions, adjusted to pH 5.8 with acetic acid, were identical, except that EDTA was absent. (iii) Ethanolic EDTA containing 80% ethanol and 0.23 M trimethylammonium EDTA (Scott & Kyffin, 1978), followed by staining with CB for 3 h at 37 °C.

4. *Nitrous acid.* 6–7 wk RTT, 11 wk RE, 11 and 14 wk RL sections were immersed either in nitrous acid (HNO_2) or a pH control for 15 min at RT then washed $\times 3$ with H_2O before staining with CB overnight at RT. The HNO_2 solution comprised equal volumes of 5% w/v sodium nitrite and 10% v/v acetic acid. The control solution contained 0.7 M NaAc (the same Na molarity as the test HNO_2 solution), adjusted with glacial acetic acid to the same pH (3.7) as the HNO_2 solution.

Experiments with glutaraldehyde-fixed RTT sections were exposed to the same treatments and stained similarly.

5. *Acetylation.* 6–7 wk RTT sections were immersed for 10 min at RT in a fresh aqueous solution of 0.5% v/v acetic anhydride in 1% w/v sodium bicarbonate (initial pH 6.9, decreasing to pH 5.9). The sections were washed $\times 3$ in H_2O and stained with CB overnight at RT.

Experiments on glutaraldehyde-fixed sections were performed similarly.

6. *Hydrogen peroxide.* 10–12 wk RTT sections were immersed in 1 vol. H_2O_2 in phosphate buffered saline (PBS) for 1 h at RT, with and without 4% v/v n-propanol as $OH^{\bullet}$ scavenger (see also periodate below). Sections were washed $\times 3$ for ~ 30 s in 0.1 M $MgCl_2$/25 mM NaAc pH 5.8 solution containing 2.5% glutaraldehyde and stained with CB at 37 °C for 3 h.

7. *Mercaptoethanol.* 10–12 wk RTT sections were immersed in 0.5% v/v mercaptoethanol for 2 h at RT in PBS solution. Sections were washed for ~ 30 s in 0.1 M $MgCl_2$/25 mm NaAc pH 5.8 solution, then fixed in 25 mm NaAc pH 5.8/2.5% v/v glutaraldehyde for 30 min. After washing $\times 3$ in 0.1 M $MgCl_2$/25 mm NaAc pH 5.8 solution containing 2.5% glutaraldehyde sections were stained with CB at 37 °C for 3 h. This procedure prevented contact of mercaptoethanol either with CB or glutaraldehyde, with both of which it reacts.

8. *Periodate.* Adult RC and 6–7 wk RTT sections were immersed in aqueous 1% w/v sodium periodate at RT for 60 min. To scavenge $OH^{\bullet}$ radicals which are generated in periodate solution, 4% v/v n-propanol (Scott et al. 1972) was used in parallel experiments. Sections were washed $\times 3$ with H_2O and stained overnight at RT.

9. *NaOH.* 11 wk RE, 11 wk RL, 12 wk RTT, 16 wk MC and 14 wk RC sections were fixed with glutaraldehyde, washed in 3 changes of 25 mM NaAc pH 5.8 and immersed in either 0.1 M NaOH or 0.1 M NaAC pH 5.8, used to provide a control level of Na^+ concentration, for 3, 4 or 6 h at 37 °C. The slides were horizontal (sections uppermost), as opposed to the usual vertical position in Columbia jars, to improve recovery of sections after the NaOH treatment, which tended to detach sections from the slides under the pull of gravity. They were washed $\times 2$ in 0.1 M NaAc pH 5.8 and $\times 1$ in 0.1 M $MgCl_2$/2.5% glutaraldehyde/25 mM NaAc pH 5.8 before staining with CB solution overnight at RT, with the slides horizontal.

10. *Urea.* 8 wk RTT sections were immersed in 1.0 M, 2.0 M, or 5.0 M urea (natural pH 6.7) at RT for 1 h, before staining with CB incorporating urea at the molarity of the test solutions, at 37 °C for 3 h.

Experiments on glutaraldehyde fixed sections were performed similarly. Other sections were immersed in urea solutions containing Triton X100 (see 11 below).

11. *Triton X100 and Triton/urea.* 10–12 wk RTT sections were immersed for 1 h or overnight at RT in 1% v/v Triton X100 solution in PBS, washed $\times 3$ in 0.1 M $MgCl_2$/25 mm NaAc pH 5.8–2.5% glutaraldehyde and 1% Triton before staining with CB for 3 h at 37 °C incorporating 1% v/v Triton.

10 wk RTT sections were immersed in solutions containing, 0.7%, 0.4% or 0.1% v/v Triton including 0.5 M or 1.0 M urea or 1% Triton in 2.0 M urea, adjusted to pH 6.7 with NaOH, for 1 h at RT, before staining with CB at 37 °C for 3 h. The staining solution contained urea and Triton at the concentration present in the test solution.

10 wk RTT sections fixed with glutaraldehyde were treated with 0.1% Triton in 0.5 M urea or 1.0% Triton in 2 M urea and stained at 3 h at 37 °C. 8 wk fixed RTT sections were immersed in 1.0% Triton containing either 1 M or 2 M urea and stained similarly.

12. *Organic solvents.* 16 wk RTT sections were immersed in (a) 80% v/v ethanol for 1 h, (b) 100% ethanol for 1 h, or (c) 100% ethanol for 1 h followed by immersion in 2:1 chloroform:methanol for a further 1 h, all at RT. They were then stained with CB at 37 °C for 3 h.

RESULTS

The conditions in which the test was performed were frequently carried through into the staining stage to avoid rearrangements or ultrastructural changes sec-

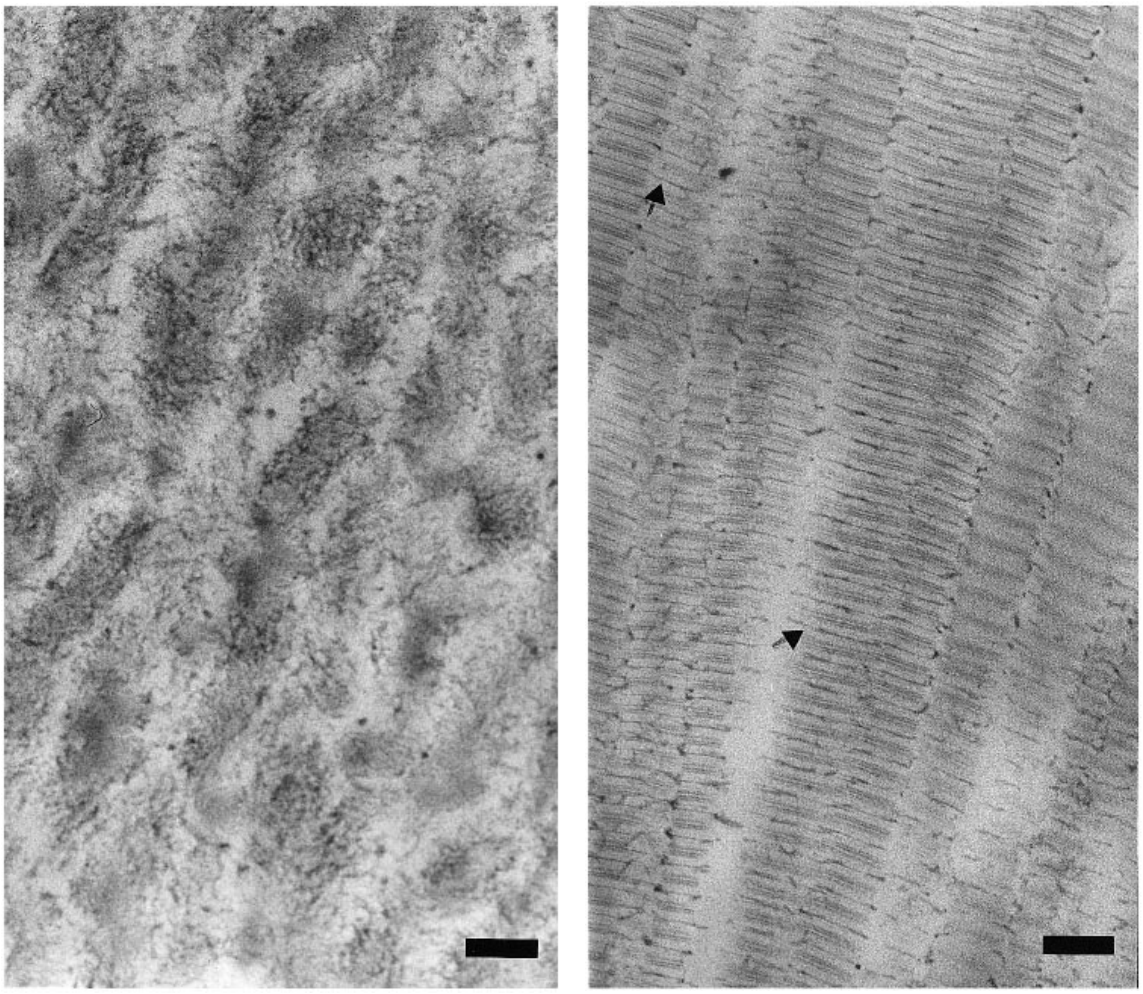

Fig. 2. Effects of changing pH on the ultrastructure of RTT. (*a*) At pH 2.0 there is gross disruption of the tissue with no shape modules visible. (*b*) At pH 8.5 there is regular ultrastructure with shape modules every D period, frequently seen bridging 2 or more fibrils (arrowed). Stained with Cupromeronic blue and STA to demonstrate AGAGs and collagen, respectively. For details see text. Bar, 165 nm.

ondary to the removal of the test reagents during staining. Details are given below and in the Materials and Methods section. Although most of the illustrations are of RTT sections, other fibrous tissue ECMs yielded identical conclusions.

1. *pH*. Between pH 5.5 and 9.5 AB-stained mature RTT showed numerous orthogonal PG filaments as bridges between STA-stained collagen fibrils. At pH 4.0 and 4.5 only occasional orthogonal bridges were visible, although the fibrils were mainly intact and D-periodicity was apparent. At pH 2.0 disruption of the whole tissue involved both collagen fibrils and PG filaments (Fig. 2). At pH 3.7 most bridges survive in *fixed* tissue in which fibrils are not disaggregated (see nitrous acid experiments, 4, below).

2. *$MgCl_2$*. Disruption of shape modules in unfixed tissues was severe in 0.5 M $MgCl_2$ compared with 0.1 M $MgCl_2$. The effect of 1.0 M $MgCl_2$ on *fixed* tissues was not dramatic.

3. *EDTA*. In aqueous treatments of unfixed RTT this caused no loss of PG bridges compared with controls. EDTA in 80% ethanol:H_2O caused noticeable disruption of AGAG structures which was probably due to 80% ethanol:H_2O rather than the EDTA (see 12 below).

4. *Nitrous acid*. In 6–7 wk RTT, disruption was considerable, both in the test and the pH control solutions. Fibrous tissue in RE and RL treated with either HNO_2 or the pH control solution showed similar losses of orthogonal PG bridges compared with nontreated tissue. Compared with unfixed tissues glutaraldehyde-fixed RTT sections retained many more bridges both in HNO_2 and pH control samples, probably more in the latter.

5. *Acetylation*. In unfixed tissues this caused a significant loss of bridges compared with controls. The effect was prevented by prior fixation with glutaraldehyde. The orthogonal AGAGs then ap-

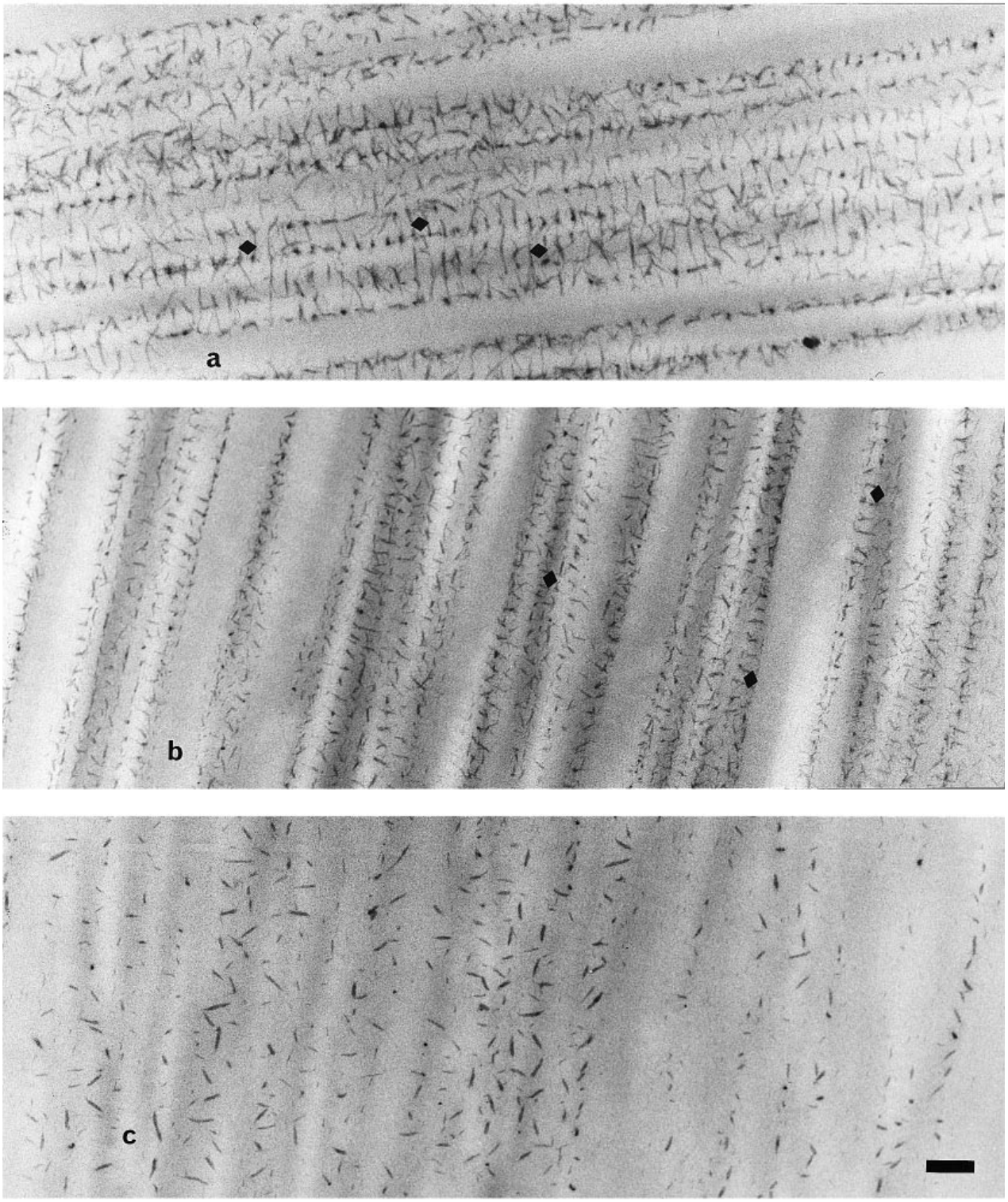

Fig. 3. Effects of acetylation on AGAG bridges in RTT. (*a*) After treatment with $NaHCO_3$/acetic acid control solution at ~ pH 6.5. The picture is normal with many orthogonal AGAG arrays and interfibrillar bridges (diamonds). (*b*) After treatment of glutaraldehyde-fixed RTT with acetylation reagent. The picture is essentially normal with numerous orthogonal AGAG arrays and interfibrillar bridges (diamonds). (*c*) After treatment of unfixed RTT with the acetylation reagent as in *b*. There are very few bridges and the picture is of more randomly oriented AGAGs than in *a* and *b*. Stained with Cupromeronic blue to demonstrate AGAGs. For details see text. Bar, 190 nm.

peared shorter and their overall pattern of distribution was somewhat simpler than for the controls (Fig. 3).

6. *Hydrogen peroxide*. At more than 1 vol. this caused general disruption of the tissue. One vol. H_2O_2 had no significant effect on the incidence of bridges. The free radical scavenger n-propanol did not modify the action of 1 vol H_2O_2, which was anyway slight.

7. *Mercaptoethanol*. In our conditions this did not

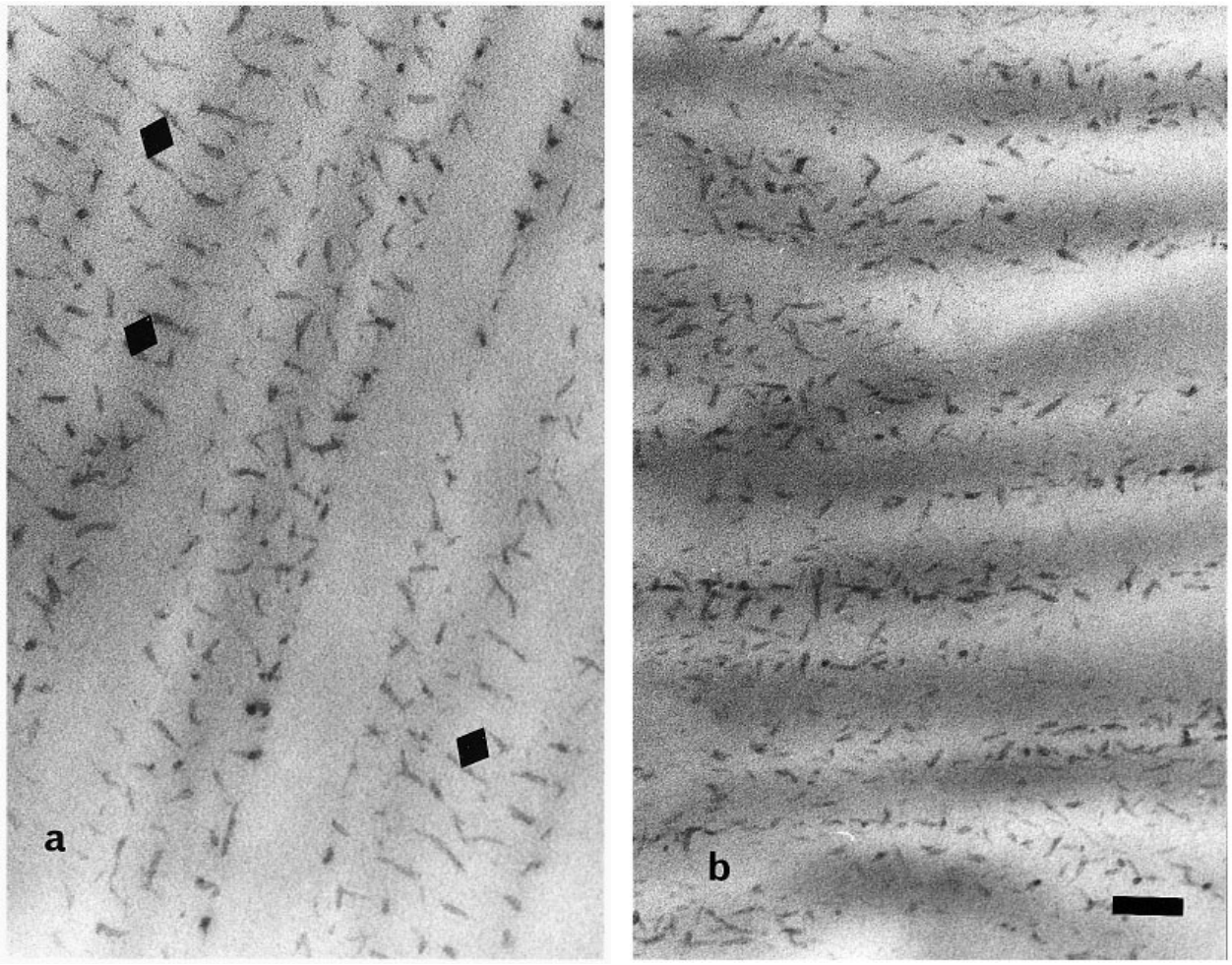

Fig. 4. The effects of periodate ($NaIO_4$) on glutaraldehyde-fixed RTT. (*a*) With n-propanol as a free radical scavenger (diamonds). There are numerous orthogonal AGAG arrays in a normal pattern. (*b*) Without a free radical scavenger. The AGAG orientations are mainly random although the positioning of many AGAGs along the fibril is regular, suggesting that the PG:collagen interface (2, in Fig. 1) was not the primary site of the free radical attack. The fragmented appearance of the AGAGs suggests rather that they were a target for the free radicals. Stained with Cupromeronic blue to demonstrate AGAGs. Bar, 90 nm. For details see text.

affect the incidence of bridges. Possibly, disulphide bridges reduced by the reagent reformed in the prestain washes that removed mercaptoethanol to prevent it encountering glutaraldehyde or CB.

8. *Periodate*. At 1.0% w/v for 60 min at RT this considerably decreased the number of bridges and broke up orthogonal arrays, presumably due to free radicals since it was prevented by the addition of n-propanol (Fig. 4).

9. *NaOH*. Very few orthogonal AGAG bridges were observed in RTT, RE, RL and MC after treatment with 0.1 M NaOH, compared with 0.1 M NaAc treatment at pH 5.8. The few filaments that remained in RTT were mainly randomly distributed (Fig. 5).

In RC, however, although a reduction in the number of orthogonal AGAG bridges was observed after treatment with 0.1 M NaOH, many bridges were still present, located at the a and c bands of UA stained collagen. PKSs bind specifically at these bands. AGAGs located in control RC at the d and e bands were absent after NaOH treatment. These AGAGs are CS/DSs (Scott & Bosworth, 1990). Practically no CB-stained filaments were observed in MC following alkali treatment.

10. *Urea*. 5 M urea caused gross disruption of unfixed tendon, after which collagen fibrils were not recognisable. 2 M urea caused severe damage, although individual fibrils and some PG bridges were observable, but 1 M urea was without serious effect and collagen fibrils and shape modules appeared normal in morphology and number. Glutaraldehyde-fixed sections showed good fibril morphology and normal statistics of bridge occurrence.

11. *Triton X100*. At 1% v/v this had no effect on the gross of ultrastructural morphology of unfixed tendon. However, when combined with urea the effects were much more striking than with either

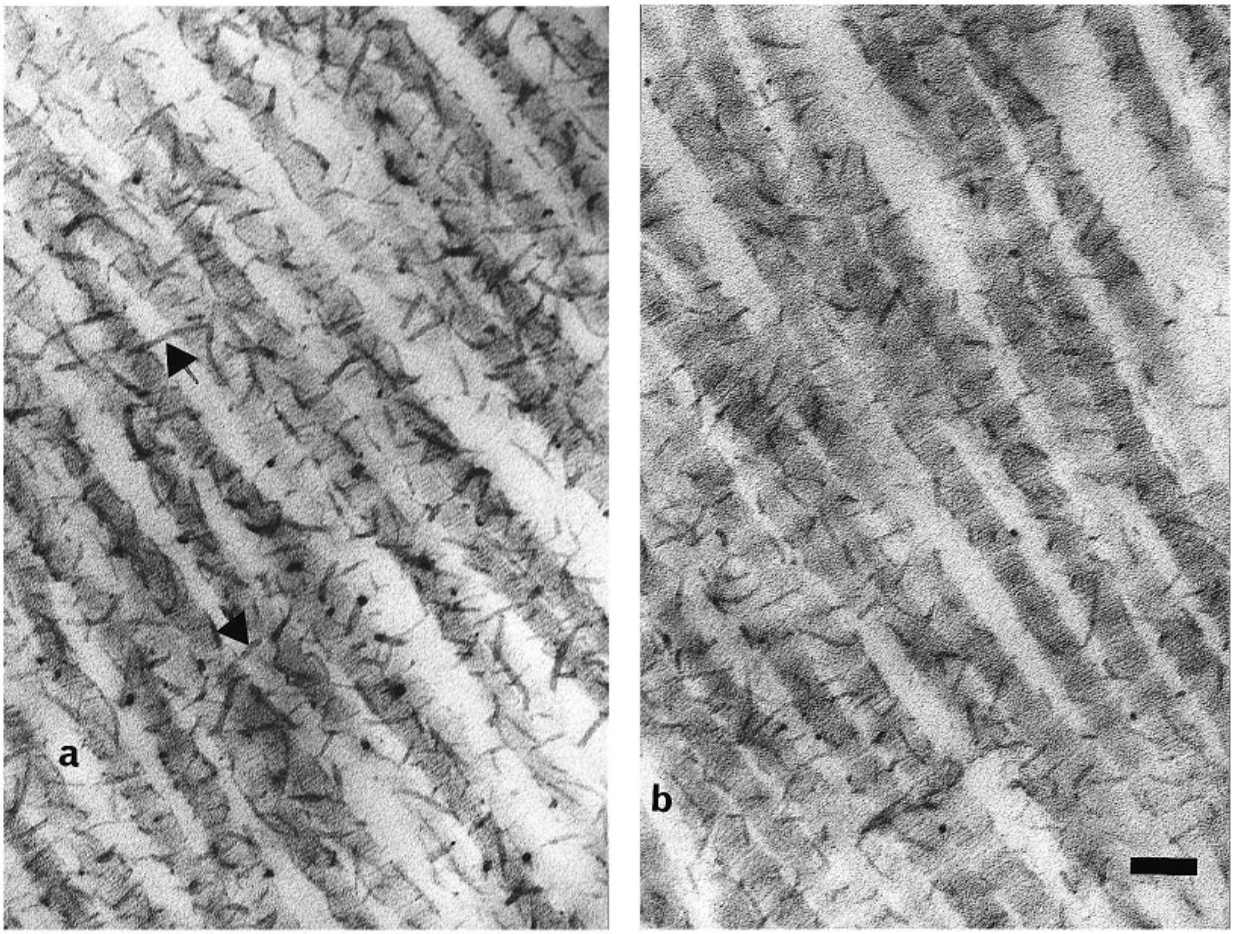

Fig. 5. Effects of treatment of glutaraldehyde-fixed rabbit corneal stroma with 0.1 M NaOH. (*a*) Control tissue treated with 0.1 M NaAc pH 5.8 (the same Na molarity as the NaOH solution). Large amounts of AGAG are seen, with numerous orthogonal arrays bridging between collagen fibrils (arrowed). (*b*) After treatment with 0.1 M NaOH. Far less AGAG is seen than in *a*, but much is ordered and orthogonally placed, bridging the collagen fibrils. Stained with Cupromeronic blue to demonstrate AGAGs and with uranyl acetate for the collagen fibrils. Bar, 85 nm.

separately. Thus 0.7% Triton X100 in 1.0 M urea caused a dramatic reduction in the number of shape modules (Fig. 6).

12. *Organic solvents*. RTT treated with ethanol (80% or 100%) showed many fewer orthogonal PG bridges compared with control tissue. Chloroform: methanol reduced the number of orthogonal bridges further, compared with ethanol (Fig. 7).

DISCUSSION

Properties of the interfibrillar bridges that emerged from this investigation are relevant to their physiological, biophysical and pathological behaviour. Ultrastructural investigations of PG:collagen interactions in the shape module are now established on a firmer phenomenological basis. The results will help to interpret future investigations of shape modules etc. on tissues treated in various ways.

A structure was proposed for the shape module (Fig. 1) in which the protein moiety of the PG associates noncovalently with the collagen fibril and the AGAG chains aggregate in an antiparallel fashion, bridging the gap between fibrils (Scott, 1992*b*). Indeed, PDSs spontaneously aggregated in solution via antiparallel AGAGs, to give a dumbbell shaped structure with globular proteins at each end, similar to that in Figure 1, as observed in rotary-shadowed electron micrographs (Ward et al. 1986). The PG proteins bind at specific binding sites in the gap zone of collagen fibrils (Scott, 1996). However, important points remain to be proved concerning the situation in tissues and some binding modalities are speculative. This investigation presents evidence for some of these interactions.

Treatments which most affected unfixed tissues involved urea, urea plus Triton, $MgCl_2$, acetylation, acid pHs, free radicals generated by periodate, and organic solvents. Those with little or no effect were

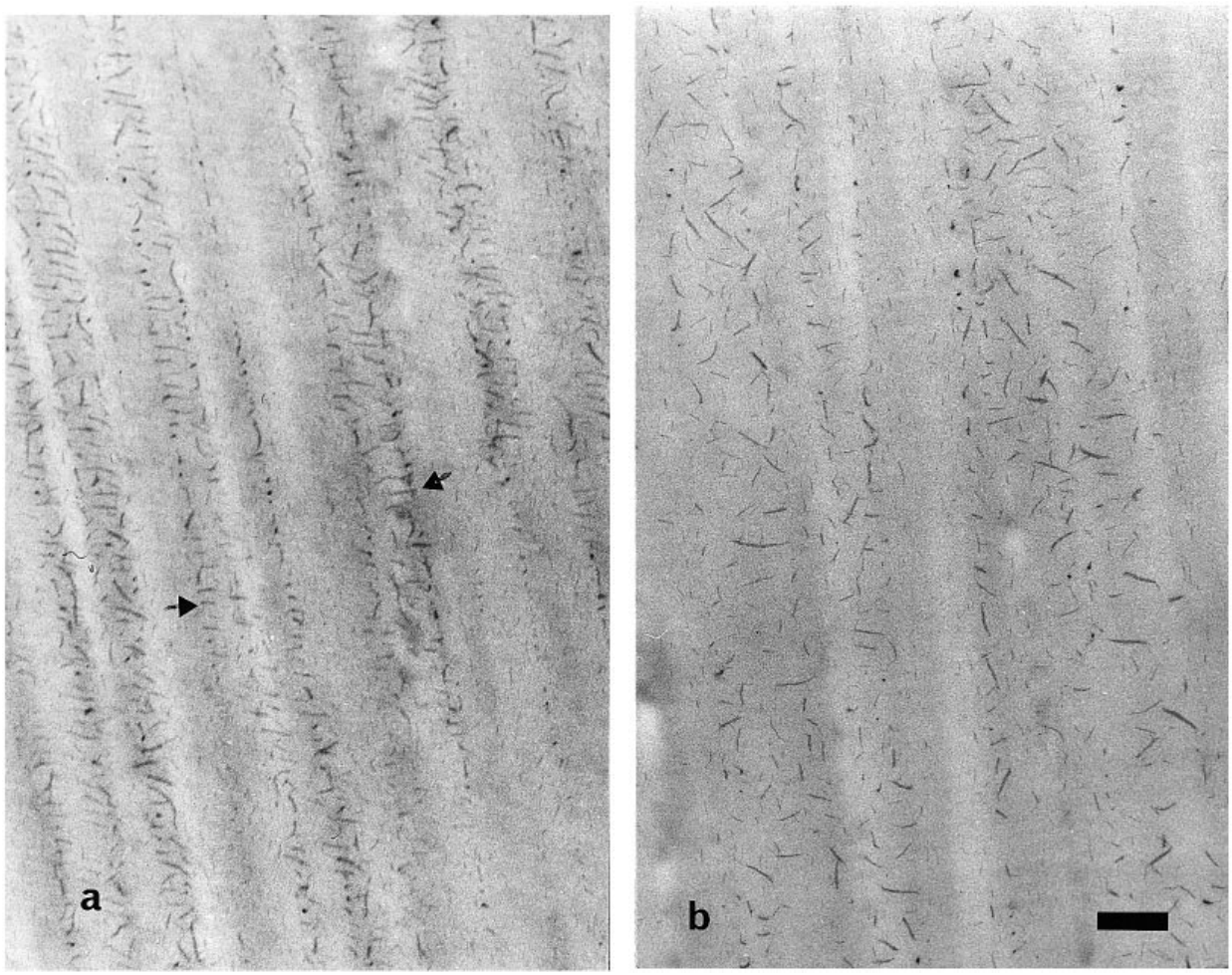

Fig. 6. Effects of urea and Triton X100 on the ultrastructure of RTT. (*a*) After immersion in 1.0 M urea. The AGAG orthogonal arrays are normal with numerous interfibrillar bridges (arrowed). (*b*) After immersion in 1.0 M urea containing 0.7% Triton X100. The picture is more random than in *a*. Many AGAGs are positioned at regular D periodic intervals, suggesting that the PG: collagen interface (2 in Fig. 1) is not the prime target of the reagent. Rather, the disruption of the orthogonal arrays suggests that the AGAG aggregates are affected by the combination of urea and Triton. Stained with Cupromeronic blue to demonstrate AGAGS. For details see text. Bar, 245 nm.

EDTA, H_2O_2, mercaptoethanol, periodate as a glycol-splitting reagent and Triton alone. Remarkably, except for the effects of free radicals produced by periodate, and cleavage of AGAG chains by NaOH, no treatment could disrupt the AGAG bridges after glutaraldehyde fixation.

Fixation

Negative results, i.e. of treatments which did not disrupt shape modules in unfixed tissues, are more readily interpreted than those from disrupted unfixed tissues, which could be due to action at one or more of 3 points in the proposed structure (Fig. 1): (1) on the collagen fibril itself, (2) on the PG protein-collagen fibril interface and (3) on AGAG aggregation. Disruption of the fibril structure at acid pH and at high $MgCl_2$ or urea concentrations (Results) was prevented by prefixing with glutaraldehyde, but this fixation also covalently stabilises the interaction between PG protein and the collagen fibril at point 2.

If the collagen fibril structure per se was not affected by a treatment, comparison of data from fixed and unfixed tissues dissects effects on the PG protein: collagen interaction (both participants in which could react with glutaraldehyde) from the AGAG self interactions, since AGAGs do not carry primary amino groups, usually assumed to be the main targets for glutaraldehyde reactivity. If, after glutaraldehyde fixation, a reagent disrupted bridges, this would imply a direct effect of the treatment on the AGAG aggregation and not on the PG protein:collagen interaction. Conversely, if glutaraldehyde fixation prevented an effect shown on unfixed tissue, at least part of the effect was exerted on the PG protein:fibril interface.

However, glutaraldehyde $-CHO$ groups might react with AGAG $-OH$ groups, producing hemi-

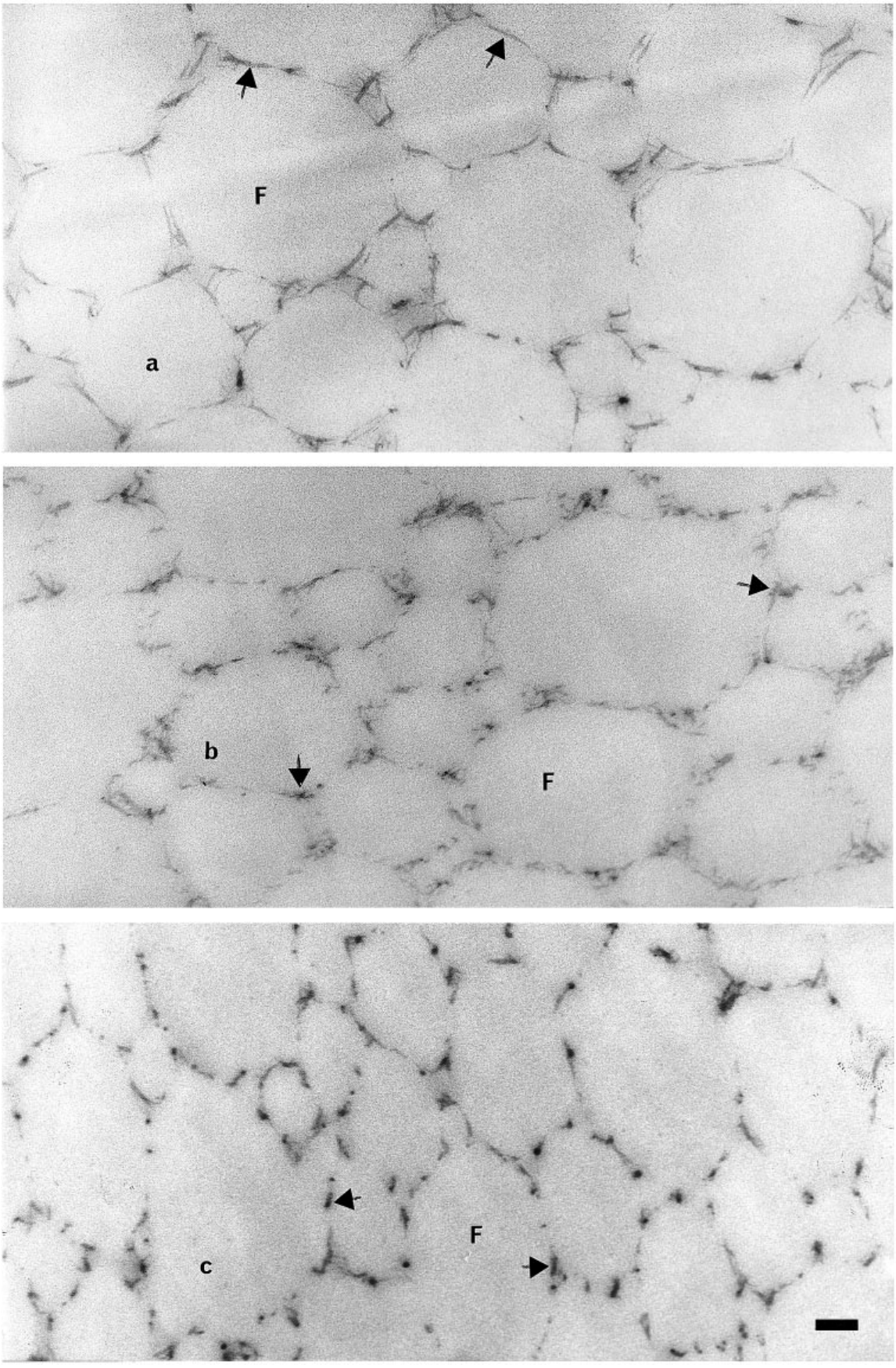

Fig. 7. For legend see opposite.

acetals. The second $-CHO$ group, distal to the hemiacetal, could react with NH_2 groups on the fibril, or with other $-OH$ groups (e.g. on the glycans, collagen fibrils or elsewhere), thus forming or stabilising supramolecular structures. Although the reaction is easily reversible and hemiacetals have not often been isolated, at least one large class of hemiacetals is stable in water, i.e. the reducing sugars (e.g. glucose), because steric factors favour their hemiacetal ring structures. Thorough washing after glutaraldehyde treatments would have ensured that few if any hemiacetals remained in our sections, but their total absence cannot be assumed and possible consequences must be kept in mind. Hemiacetals can convert to acetal, providing a second $-OH$ group is sufficiently close to the first to permit the formation of the cyclic acetal. Acetals could form from the 2, 3 glycol group in the uronic acid moiety of e.g. dermochondan sulphate. Acetals are stable in water but would not be expected to form in the practically neutral aqueous environment. Since NaOH removes almost all AGAG from fixed tendons etc. (Results), few if any of these products could have been available to fix AGAG chains in the shape modules.

The fact that glutaraldehyde fixes PG:collagen interactions indicates that NH_2 groups in one participant are close to NH_2 groups in the other, and biochemical evidence backs this up (Scott et al. 1997). This does not prove that they are involved in vivo. The effects of acetylation on unfixed tissue implicate NH_2 more directly (see below). Reaction of glutaraldehyde with NH_2 groups hinders subsequent acetylation and reaction with nitrous acid.

It is emphasised that fixative action of glutaraldehyde it is not an essential determinant of the shape module as seen and investigated by us and many others. Orthogonal arrays are observed in large numbers in unfixed tissues, as well as in tissues fixed with formaldehyde (data not shown).

PG protein:collagen fibril interactions (*2 in Fig. 1*)

Neither mercaptoethanol nor hydrogen peroxide caused important damage to the bridges (in unfixed tissues). This implies that oxidisable and reducible groups play little part in stabilising the PG bridge structure. Thus the intact (i.e. not reduced to SH or oxidised to sulphonic acids) disulphide bridges that—are present in PDS are not essential to the stability of shape modules and the PG protein interaction with collagen fibrils is not broken by cleavage of decoron disulphides. This result should be compared with those of P. G. Scott et al. (1986) that decoron disulphides were essential in inhibiting collagen fibrillogenesis, in vitro. Possibly disulphide bridges are less important once the tissue structures have been formed.

Reduced alkylated decorin spontaneously aggregated in solution via its AGAG chain to form dumbbell shaped structures similar to those in the shape modules (Fig. 1) (Ward et al. 1986), suggesting that the PG disulphides were not essential in the generation of antiparallel structures of the kind suggested to be present in tissues.

Ionic interactions in the stability of shape modules

PG interfibrillar bridges in unfixed mature RTT are stable in mildly acidic ($>$ pH 4.5), neutral and alkaline (pH $<$ 9.5) conditions. Since mature RTT bridges contain predominantly PDS (Scott et al. 1981), we conclude that PDS interfibrillar bridges are stable between pH 4.5 and pH 9.5. $-COO^-$ and $-NH_3^+$ groups do not significantly change their states of ionisation within this pH range, although histidine imidazoles would. Thus histidines are not vitally involved in the bridge interactions and conversely, NH_2 and $-COO^-$ groups on other aminoacids, AGAGs etc are not excluded. Ionic attractions between specific aminoacids were postulated to occur in tissue decoron:collagen fibril association (Scott, 1996).

In more acidic conditions the tendon breaks down and in more alkaline conditions dermochondan AGAGs are chemically cleaved from their PGs (see below). PKS bridges are stable even at pH 13 (see below) in *fixed* tissues.

The action of 0.5 mM $MgCl_2$ in disrupting shape modules is strong evidence for an ionic component of the interaction between PG and collagen. The effects were probably on the collagen:PG protein interface since the AGAG bridges remained intact in tissue fixed before treatment with $MgCl_2$, assuming that glutaraldehyde did not fix AGAG. At low (0.1 M)

Fig. 7. Effects of organic solvents on AGAG structures in RTT. Cross sections of collagen fibrils, stained with Cupromeronic blue to demonstrate AGAGs. For details see text. (*a*) Control untreated by organic solvents. AGAGs (arrowed) are long and segmented. Their combined lengths frequently entirely encircle a fibril. (*b*) After immersion in 80% v/v ethanol H_2O, AGAG segments are much shorter, frequently seen merely as clumps of stained material (arrowed). (*c*) After immersion in chloroform:methanol 2:1 v/v. The AGAG aggregates (arrowed) are more compact than in *a* and *b*. Considerable stretches of fibril perimeter lack any associated AGAG. Many isolated punctate AGAGs are seen, compared with *a* and *b*. F, collagen fibril. Stained with Cupromeronic blue to demonstrate AGAGs. Bar, 82 nm.

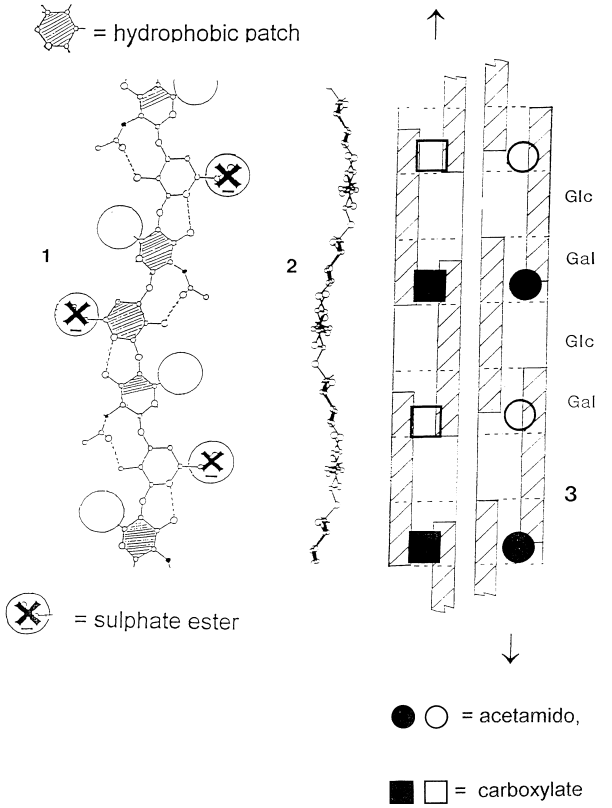

Fig. 8. Model of proposed structure in the AGAG aggregates which comprise the orthogonal interfibrillar bridges in ECM. (1) Plan view of the polylactose AGAGs (CS, DS, keratan sulphates) as 2-fold helices, with the hydrophobic patches stretching across 3 sugar units. There is a gentle wave in the tape-like molecule in the plane of the paper. (2) The tape in side view, again showing a gentle wave in the plane of the paper. (3) How 2 such molecules can duplex together is shown, with the hydrophobic patches (cross hatched) coming together and thus hydrophobically bonding to each other in water. The gentle waves in planes perpendicular to each other (as in (1) and (2)) are matched perfectly in this duplex providing only that they are antiparallel to each other. Each sugar unit is delimited by a dotted line. The open symbols for acetamide and carboxylate are on the same side of the duplex, able to H-bond to each other. The closed symbols are on the opposite side of the duplex, e.g. below the plane of the paper, equally able to H-bond to each other. Keratan sulphate carries CH_2OH groups in the position in which CS and DS carry carboxylate groups.

$MgCl_2$ concentrations orthogonal AGAG arrays remained intact in unfixed tissues. At high molarity (1.0 M) in fixed tissues the AGAG bridges are stable, confirming that AGAG aggregation is not primarily ionic. Glutaraldehyde prevented break-up of the shape modules, probably by crosslinking lysine $-NH_2$ groups in the collagen and the PG.

Aqueous EDTA did not disrupt PG bridges. This implies that divalent cations, e.g. Ca^{2+} which associate with the AGAG chains in vivo because of their polyanionic nature, are not critically involved in the AGAG bridge stability. Although 80% v/v ethanolic EDTA (Scott & Kyffin, 1978) disrupts PG bridges in mature RTT (Results) the effect is due to the 80% ethanol (Fig. 6) rather than to EDTA. Data on PG:collagen ultrastructures should be interpreted cautiously after using this reagent.

Acetylation caused considerable disruption of unfixed tissues which was largely prevented by glutaraldehyde fixation (Fig. 3). Under the very mild conditions (RT, bicarbonate buffer) only primary amino groups would undergo high levels of acetylation. This suggests that NH_2 groups are involved in the stability of the shape modules (see discussion on fixation, above) at positions 1/2 (Fig. 1) and that NH_2 groups may play a role in the positioning of AGAG chains across the collagen fibrils. The protective action of glutaraldehyde against acetylation could be due to fixation of the protein:protein interactions and/or blocking of NH_2 groups to reaction with acetic anhydride.

Unfixed tissue was severely damaged in HNO_2 solution, but it was similarly disrupted in control solutions of identical pH, thus preventing any specific conclusions about the effects of HNO_2 on unfixed tissue.

AGAG bridges in fixed tissues were only slightly disrupted by HNO_2. Many NH_2 groups would have reacted with glutaraldehyde, hindering reaction with nitrous acid.

AGAG-AGAG interactions (3 in Fig. 1)

Chondroitin sulphate and keratan sulphate are both based on a polylactose chain (Fig. 8), with varying sulphation patterns (Scott, 1994). The polylactose chains can form 2-fold helices in aqueous solution in which hydrophobic patches are present in similar positions alternating on either side of the chain (Fig. 8). These promote aggregation between 2 or more chains (Fig. 8) by hydrophobic bonding, in water. This potential also exists in dermochondan chains (Scott et al. 1995) because similar hydrophobic structures are present, slightly modified by the replacement by L-iduronic acid of some of the D-glucuronic acid in chondroitin sulphate.

The proposed mechanism of aggregation includes hydrogen bonding as well as hydrophobic bonding between neighbouring molecules (Fig. 8). H-bonding is usually weak in water because H_2O competes both for donor and acceptor groups. These attractive forces are opposed by repulsive forces between the anionic charges of the aggregating AGAGs.

Triton X100 per se did not disrupt AGAG bridges. This suggests that hydrophobic bonding alone does not stabilise AGAG aggregates, since nonionic detergent:AGAG interactions could substitute for

hydrophobic AGAG:AGAG interactions, thus competing for the obstructing AGAG–AGAG association.

Urea at high concentrations (5 M) disrupted the tendon structure. Toole & Lowther (1968) used 6 M urea at 60 °C to liberate PDS from tissues. At lower concentrations (~ 1.0 M) damage was limited and many shape modules persisted (Fig. 6). 1.0 M urea could elute (i.e. dissociate) free dermochondan sulphate from dermochondan sulphate covalently linked to a support (Fransson, 1976).

When Triton X100 was added to low urea concentrations which had no effect in themselves the combined effects were dramatic (Fig. 6). Since urea breaks H-bonds in secondary and tertiary structures this suggests that combined hydrophobic and H-bonding stabilises the shape modules and that both must be weakened (in the conditions of these experiments) to disrupt the AGAG bridges.

After mild Triton-urea treatment fewer bridges were seen, but many regular D-spaced PGs remained (Fig. 6) suggesting that PG-collagen interactions persisted and implying that more than hydrophobic/H-bonding is involved in the PG:collagen binding. Salt links are also implicated (see $MgCl_2$ above). Glutaraldehyde fixation prevented disruption of bridges by 2 M urea/1% Triton, which otherwise grossly damaged the tissue. If glutaraldehyde did not fix the AGAGs (see above), the urea/Triton treatment was not able to disrupt AGAG bridges.

Organic solvents disrupted the AGAG (i.e. PDS) interfibrillar bridges of mature RTT. The effect is particularly clear in pictures of cross sections of collagen fibrils (Fig. 7). Analysis of this result is compatible with the idea that hydrophobic and hydrogen bonding between AGAG chains promotes their aggregation and hence the formation of bridges between collagen fibrils.

The number of orthogonal bridges remaining after treatments correlated with the polarities of the solvents. Water is more polar than ethanol which is more polar than the chloroform:methanol mixture. Maximum association between AGAG chains occurred in aqueous environments (Fig. 7).

In less polar (e.g. ethanol) conditions, hydrophobic bonding is ineffective. With decreasing interaction between hydrophobic patches of AGAG chains, bonding in the aggregate weakens and the chains tend to dissociate.

Electrostatic interactions strengthen as the dielectric constant of the solvent decreases, and this is marked in the transition from water ($\epsilon = 80$) to ethanol ($\epsilon = 25$). Thus increased electrostatic repulsion in ethanol destabilises the aggregate, as does the weaker hydrophobic bonding. In nonaqueous solvents H-bonding solute molecules is stronger, since there is no competition from H_2O, but this was not enough to compensate for decreased hydrophobic bonding and the increased electrostatic repulsion between participants, with a consequent loss of interfibrillar bridges.

Disruption of the orthogonal arrays by organic solvents was paralleled by an increase in the proportion of axially oriented AGAGs (not shown).

Periodate in the presence of 4% v/v n-propanol caused only slight loss of bridges from unfixed tissues, although many of the glycol groups in dermochondan sulphate would have been oxidised to dialdehydes. Possibly these aldehydes link with nearby groups ($-NH_2$, $-OH$ or $-CHO$) on AGAGs or proteins, thus maintaining an aggregated structure.

However, in the absence of n-propanol as a free radical scavenger, there was considerable disruption of AGAG structures (Fig. 4). Free radicals, particularly OH, generated by periodate (Scott & Page-Thomas, 1976), are particularly destructive of polyuronides such as dermochondan sulphate. Propanol is an effective OH scavenger (Scott et al. 1972). H_2O_2 did not behave like periodate, there was no effect with or without n-propanol.

Ultrastructural localisation of keratan sulphate following NaOH treatment

0.1 M NaOH treatments of glutaraldehyde-fixed tissues showed species and tissue-specific effects. Of the tissues examined only RC retained orthogonal PG bridges after treatment. These were identified as PKS, since they were at the a and c bands of the collagen fibrils (Scott, 1988). Thick mature corneal stromas of larger animals (rabbit, cow, human, etc.) contain both PDS and PKS. PDS in corneal stroma associates at the fibril d and e bands (Scott, 1988). Their AGAGs were eliminated by NaOH treatment (Results). MC, which is very thin, does not contain PKS but only PDSs, unlike thicker corneas (Scott & Bosworth, 1990). Compatible with this, no bridges were left in MC after NaOH treatment, all the AGAGs having been removed. This finding confirms previous results obtained by biochemical assay and enzyme digestion that mouse cornea is essentially free of keratan sulphate of the kind typically found in larger animals (Scott & Bosworth, 1990). We conclude that PKS interfibrillar bridges in fixed cornea are stable at high pH, whereas PDS bridges are not. It is remarkable that PKS AGAG bridges resist a pH as high as 13.

AGAGs from fibrous tissues of tendon, ear and lung were eliminated by NaOH treatment (Results), compatible with the fact that they are attached to PDS and PCS, but not PKS.

The differing stabilities of the PDS and corneal PKS bridges derive from the linkage structures between the core proteins and AGAG chains. PDS contains O-linkages (AGAG-xylosyl-O-serine-core protein) and N-linkages are present in corneal PKS (AGAG-GlcNAc-N-asparagine-core protein).

OH^- ions cleave the O-link to serine, but not the N-link to asparagine from GlcNAc. Cleavage of the O-link releases the dermochondan sulphate chain into the surrounding solution. The interfibrillar bridge is thereby removed.

Thus NaOH treatment provides a relatively crude and simple (but based on well established chemistry) method of distinguishing between PDS and PKS and their ultrastructural localisations in corneas.

Conclusion

The picture of the shape modules which emerges is of resistant structures, unaffected by oxidation, reduction and wide variations in pH or Ca^{2+} concentrations. Free radicals damaged them, otherwise harsh conditions that would not occur in vivo were needed to disrupt PG bridges. Nonpolar solvents probably dissociated AGAG bridge aggregates while 0.1 M NaOH chemically removed dermochondan chains from the tissues. Even after 0.1 M NaOH treatment, corneal PKs interfibrillar bridges were still extant in fixed tissue.

The marked stabilising effect of glutaraldehyde on the orthogonal AGAG arrays revealed by these experiments was noteworthy. Possibly AGAG aggregates are sufficiently stable per se to resist $MgCl_2$, acid pH, urea and organic solvents, providing the shape module structure is fixed by glutaraldehyde at points 1 and 2 (Fig. 1). Alternatively glutaraldehyde may fix dermochondan sulphate in a manner not yet clear. Thirdly, there may be interactions within the shape module that can only be speculated about, e.g. the disruptive effect of organic solvents hints at the extraction of a lipid component. The fixative effect of glutaraldehyde might suggest an NH_2-carrying species is involved.

However it is achieved, considerable advantage must accrue to the organism in possessing a shape-maintaining capacity that is unaffected by big changes in the milieu interieur.

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