Virchows Archiv (1994) 424:89–98

ORIGINAL ARTICLE

John E. Scott · Timothy R. Bosworth
Alison M. Cribb · Axel M. Gressner

The chemical morphology of extracellular matrix in experimental rat liver fibrosis resembles that of normal developing connective tissue

Received: 2 August 1993 / Accepted: 4 December 1993

Abstract The time course of development of extracellular matrix (ECM) in experimentally induced fibrosis (thioacetamide administration followed for 12 weeks or bile duct ligation for 8 weeks) in adult rats was examined by light and electron microscopy, using Alcian blue or Cupromeronic blue staining for sulphated proteoglycans (PGs) in critical electrolyte concentration techniques. Proteodermatan sulphate (PDS) was regularly observed at the gap zone of the collagen fibrils. Morphometry of uranyl acetate-stained collagen fibrils, polarity of their banding patterns (a–e), statistics of d/e band occupancies by PDS, and lengths and thicknesses of PG filaments were quantified. Biochemical analyses showed that the ECM components collagen, hyaluronan, chondroitin and dermatan sulphates increased by 5–10 fold, roughly in parallel, as did heparan sulphate and DNA. Water and lipid contents also increased sharply. Thioacetamide treatment was much slower than bile duct ligation in producing fibrotic changes of equal severity. Sulphation of anionic glycosaminoglycans (AGAGs) decreased with increasing severity of fibrosis. Biochemical and ultrastructural methods correlated well. The large increase in dermatan sulphate was quantitatively as expected, given that it is collagen fibril surface-associated, and there was an increase of collagen content together with a decrease in fibril thicknesses. The increase in DNA reflected the marked increase in cell numbers in fibrotic livers. The chemical morphology of the new connective tissue closely resembled that in e.g. developing young tendon, in that fibrils were thinner, and AGAG levels were higher. The collagen fibrils were often disarranged, rather than ordered and parallel as in normal ECM. No other indication of abnormality in the new ECM was obtained.

J.E. Scott (✉) · T.R. Bosworth · A.M. Cribb
Department Chemical Morphology, Manchester University, Manchester M13 9PL, UK

A.M. Gressner
Department Clinical Chemistry, Philipps University, Marburg, Germany

Key words Chondroitin sulphate · Collagen
Dermatan sulphate · DNA · Hyaluronan
Lipid · Proteoglycans · RNA · Cupromeronic blue
Alcian blue · Critical electrolyte concentration

Introduction

Fibrosis is the inappropriate laying down of connective tissue (Gressner 1991). "Inappropriate" includes "in the wrong place" and "in too large a quantity". The fibrotic collagens, proteoglycans (PGs) etc. are of known types, but knowledge of their supramolecular organisation was lacking. Recently, new methods have elucidated quantitative and qualitative relationships *in tissues* between collagen fibrils and PGs (Scott 1988), and between anionic glycosaminoglycans (AGAGs) (Scott 1992).

Collagen fibrils are polar, in that collagen molecules in the fibril all point in the same direction. The polarity of a particular fibril is established by reference to the staining pattern revealed by heavy metals, which is in turn determined by the way collagen molecules align alongside each other in quarter-staggered arrays. This pattern, designated a–e, looks somewhat like a barcode. In normal tissue a given fibril has approximately equal numbers of neighbours with parallel or anti-parallel a–e patterns, i.e. with the same or opposite polarities. This arrangement may decide whether collagen fibrils fuse to bigger fibrils. In prinicple only fibrils with parallel polarities should fuse (Scott and Parry 1992).

Collagen fibrils bind macromolecules characteristic of extracellular matrix (ECM). One association observed in all soft (non-mineralised) connective tissues is that of small PGs with specific binding sites along the collagen fibrils. Small PGs consist of a globular protein to which one or two AGAG chains [chondroitin, dermatan and keratan sulphates (CS, DS & KS)] are attached. Electron histochemistry showed that proteodermatan sulphate (PDS) associated with collagen fibrils at the d and e bands and proteokeratan sulphate (PKS)

was at the a & c bands. The four binding sites were occupied by different PGs, giving rise to the hypothesis of one binding site: one PG. The ratio of occupancies d/e by PDSs and of a/c occupancies by PKSs showed species and tissue variation (Scott 1988).

A stoicheiometric relationship between the concentration of DS and the total surface area of the collagen fibrils (Scott 1984) was as expected, given that PDS associates regularly with the fibril periphery. The ratio of concentrations of DS to collagen therefore varied inversely with the average fibril diameter. During development and maturation quantitative relationships between DS and collagen changed (Scott 1986) as collagen fibril diameters changed.

The most recently discovered type of molecular recognition in ECM is that between the AGAGs (e.g. between DS and DS) which probably plays a vital role in organising the collagen fibrils vis-a-vis each other. PDSs are bifunctional cross-linking molecules that bridge and tie between collagen fibrils, the protein cores attaching to the collagen, as described above, and the AGAG filamentous aggregates bridging the interfibrillar space (Scott 1992a). Morphometry of the stained AGAG filaments provides information about the number of AGAG chains in the filament (Scott 1992b).

Our biochemical and ultrastructural study compares fibrotic tissue induced by bile duct ligation or by thioacetamide administration with normal connective tissue. A particularly relevant comparison is with young tendon. This study asks if the connective tissue of experimental liver fibrosis is "good", according to the above guidelines. Data were obtained on one or two aliquots from single rat livers, allowing estimates of individual variation to be made.

Materials and methods

Treatment of rats

Sprague Dawley rats (Lippische Versuchstierzucht, Exertal, Germany) were kept under constant lighting with a 12 h dark/light cycle and a normal laboratory chow diet. Liver fibrosis was induced either by thioacetamide administration or bile duct ligation.

One group (6 males, 2 females, mean initial body weight 205 g) received 0.3 g/l thioacetamide (Merck, Darmstadt, Germany) in drinking water to which they had unrestricted access (Gressner et al. 1977; Muller et al. 1988) for up to 3 months. The daily intake of thioacetamide was about 25 mg/kg body weight (Zimmerman et al. 1987). At 4-week intervals rats were anaesthetised with diethyl ether, blood was taken from the inferior vena cava, the liver was excised, rapidly frozen in liquid nitrogen, and stored at −80° C until required. Control rats ($n=2$) received tap water without thioacetamide.

In the other group, secondary biliary cirrhosis was induced by prolonged bile duct obstruction (Kountouras et al. 1984; Trams and Simeonidis 1957). Rats (8 males, mean initial body weigth 350 g) were laparotomised with an incision of 1 cm length under light ether anaesthesia. Double ligation of the common bile duct was followed by section between the two ligatures. Control animals were operated similarly but without ligation or section of the bile duct (sham-operation). At 2-week intervals after operation rats were killed and the livers were processed as described above. All procedures involving rats were performed according to the guidelines on the use of animals for biomedical research imposed by the German government.

Groups of animals were labelled L for ligated, T for thioacetamide-treated, C for control and 1, 2 etc. according to the sequence in which they were killed. Thus T3C is the control for the third group of thioacetamide-treated animals killed at 12 weeks (see Tables 1 and 2 for complete lists).

Biochemistry

The content of connective tissue polymers in normal liver is very low and the level of interfering substances is very high. Analytical techniques that work with typical connective tissues gave quite inaccurate results on liver, when tested for recoveries of added polymers. Wherever possible we used internal standards. Original tissue concentrations were calculated from the expression (standard)/[(analysand + standard) − (analysand)] times (analysand), where "standard" denotes the known amount of reference compound added.

Heavy losses of added *free* hydroxyproline (hyp) observed during acid hydrolysis to liberate hyp from papain digests of liver are unlikely to reflect accurately the fate of peptide-bound hyp in liver digests. A papain-digested pure gelatin of known hyp content was therefore used as internal standard, added to the tissue papain digest before acid hypdrolysis. Losses of hyp from the added gelatin peptides should be similar to those from the liver collagen, since both have been digested with papain and are therefore in similar molecular environments. We are not aware of prior use of this principle.

Two assays of HS were performed in parallel. One measured 2-sulphamato 2-deoxy glucose by fluorimetric assay of the nitrous acid cleavage product of the polymer (Scott 1979) using a heparin standard. The other used heparinase III to produce thiobarbiturate-reactive chromogens, measured exactly as the chondroitinase chromogens (Scott and Bosworth 1990), using *N*-desulphated *N*-acetylated heparin as standard. The two assays measure the two states of the HS hexosamine, and their sum should equal total HS, in principle.

All lyase products were assayed by the same procedure (Scott and Bosworth 1990). Only one buffer stock and two sets of reagents were used in five enzyme digestions and seven assays.

Materials

Hyaluronan lyase (streptomyces, EC 4.2.2.1), chondroitin lyase ABC (*P. vulgaris*, EC 4.2.2.4), heparinase III (flavobacterium, EC 4.2.2.8) and papain (crystalline, EC 3.4.22.2) were from Sigma (Poole, UK). Chondroitin lyase AC II (arthrobacter, EC 4.2.2.5) was from ICN High Wycombe, UK. Diaminobenzoic acid dihydrochloride 99% was from Aldrich (Gillingham, UK). Other chemicals were best obtainable grades.

Methods

For *defatting* and *drying*, 8 ± 4 g tissue (comprising half of each of the four lobes) was homogenised 8× in 2.5 vols chloroform/methanol (2:1) with centrifugation after each homgenisation. The supernatants were amalgamated, and an aliquot (~40 ml) was evaporated to dryness in a fume cupboard at room temperature for 48 h. The lipid residue was weighed. The tissues were similarly dried for ~18 h, until no odour of solvent was perceptible, and then weighed.

Papain digestions were in 0.6 M ammonium acetate buffer, pH 6.0 containing 0.05 M mercaptoethanol. Tissue was heated at 100° C for 25 min in buffer-mercaptoethanol. After cooling to 65° C, papain was added (total 25 mg/g dry tissue) in three portions, the second after 6 h digestion and the third at 24 h. At 30 h digests

were heated to 100° C for 25 min, cooled to room temperature and extracted 1 × with 1 vol chloroform, which was removed by centrifuging.

For *polyanion recovery*, Cetylpyridinium chloride was added at 300–400 mg/g dry tissue to the papain digest, in 3 vols H_2O, to a final ammonium acetate concentration of 0.15 M. After 18 h at 4° C the precipitate was centrifuged at 4° C, and converted into the sodium salt (Scott and Bosworth 1990).

Hydroxyproline was determined (Woessner 1961) on a freeze-dried aliquot of the papain digest supernatant after polyanion recovery, hydrolysed in 5.5 M HCl for 16 h at 105° C. To an aliquot of papain digest a known amount of internal standard was added before freeze-drying, and the total hyp after hydrolysis was compared with that of an identical aliquot of papain-digested tissue without internal standard (see above). The internal standard was of collagenous peptides from gelatin digested as above with papain, freeze dried to remove ammonium acetate and mercaptoethanol. The hyp content of this standard was 10.6% w/w. Chloramine T was used at twice the concentration of the original method. Cetylpyridinium chloride did not affect the hyp determination.

Hexosamines were determined by the Elson-Morgan reaction (Bitter and Muir 1964) and fluorimetrically (Scott 1979).

Nucleic acids (NA) were determined on the recovered polyanion, by UV absorption at 259 nm for total nucleic acids, and by fluorimetry of the diaminobenzoic acid product (Kissane and Robins 1962) for DNA, with salmon testes DNA (Sigma type III) as standard. RNA was calculated by difference, assuming the molar extinction of nucleic acid was 6000 for both DNA and RNA.

AGAGs were recovered after removal of NA, which constituted >90% of the polyanion. Aqueous trichloroacetic acid (20% w/v, 0.2 vol) was added to polyanion solution (all solutions at 0° C), mixed and after 10 min at 0° C, centrifuged at 0° C to remove precipitated NA. Over 95% of the NA was in the precipitate. Trichloroacetic acid was removed by extracting with 3 × 1 vol. diethyl ether. Remaining polyanions were recovered as ammonium salts by adding 3 vols of 1 M ethanolic ammonium acetate, leaving at 4° C overnight, centrifuging, washing in ethanol and ether, and air drying. The internal standards chondroitin-4-sulphate and *N*-desulphated *N*-acetylated heparin were added to aliquots of papain digest. *N*-Desulphated, *N*-acetylated heparin, used as standard for the heparinase III digestions (a gift from Dr. L-A Fransson of Lund, Sweden) was the preparation used previously (Scott and Tigwell 1978). HA internal standard was added before NA removal, or before lyase digestion.

Lyase digestions were in 0.3 M ammonia-acetic acid buffers, pH 8.0 at 37° C for 6 h for chondroitinases and heparinase III, and pH 5.0 at 60° C for 6 h by hyaluronan lyase. About 0.002 units of chondroitinase/μg CS(DS), 0.5 units hyaluronan lyase/μg HA, or 0.008 units heparinase III/μg HS were used. Unsaturated uronic acids were then assayed by a periodate-thiobarbiturate procedure (Scott and Bosworth 1990). Three 12.5 μl aliquots containing the thiobarbiturate product in 50% v/v dimethyl sulphoxide-H_2O were injected into a 5 μl loop, and the optical density was measured at 551.3 nm in an ACS uv-vis detector, model 750/11/AZ.

Sulphate-ester: carboxylate ratios in polyanions were determined by cetylpyridinium or dimethyl octadecylammonium titration (Scott 1960).

Electrophoresis was in 0.1 M HCl (Wessler 1970) on cellulose acetate strips (Scott and Bosworth 1990).

Critical electrolyte concentrations (CECs) were determined on total polyanion separated on strips electrophoresed in 0.1 M HCl, using Alcian blue or Cupromeronic blue in $MgCl_2$ solutions.

Alcian blue – CEC staining (Scott and Dorling 1965) of fresh frozen tissue sections was according to Haigh and Scott (1986).

Cupromeronic blue staining at 0.3 M $MgCl_2$, uranyl acetate staining, enzyme digestions of tissue sections, and electron microscopy were according to Haigh and Scott (1986).

Results

Animals and characterization of the fibrosis

Increases in body weight were very slow (thioacetamide) or negligible (bile duct ligation) during induction of fibrosis. There was no mortality among the animals. After 2 weeks, bile duct ligation generated focal cell necrosis associated with portal bile duct proliferations and mild perisinusoidal and perivenular fibrosis. After 4 weeks periportal fibrosis was observed, which became more prominent in the following 2 weeks. After 8 weeks, massive proliferation of the bile ducts with large extended lumina in conjunction with septal fibrosis and cirrhotic nodules was apparent.

Peroral application of thioacetamide resulted in septal fibrosis with initial cirrhotic nodules after 8 weeks. After 12 weeks small cirrhotic nodules with prominent periportal and peicentral connective tissue deposition was observed.

Feulgen staining of DNA showed far more nuclei in the ligated livers than in thioacetamide-treated livers, which in turn showed more than in the control livers.

Liver Biochemistry

Water content (=loss on drying through solvents) increased by 50–60% in 6–8 weeks after ligation, and by 40–50% in thioacetamide-treated livers (Table 1).

Lipid content (per g dry wt) increased by up to 30% in the ligated livers, and by much more in the thioacetamide-treated livers, one animal showing a 300% rise after 4 weeks (Table 1).

Nucleic acid (per g dry wt) determination showed that total NA increased by over 70% in the ligated livers and by less in the thioacetamide-treated animals. *DNA* increased dramatically in the ligated liver. *RNA* was approximately constant throughout (Table 1). The ratio *DNA/RNA* increased 20-fold in the 2-week-ligated liver, after which it declined to 5- to 7-fold the control level. By contrast, the increase in thioacetamide-treated livers was progressive over 12 weeks, to about 20 times the control values.

Collagenous peptides (gelatin digest) and GAGs were added to the papain digest and carried through the whole procedure. *Recoveries* were: peptide-bound hyp, 109% ($n=10$); HA, 77%; CS, 91% ($n=10$); DS, 107%. Adequate models for HS were not available. Heparin added to the papain digests was 61% recovered and

Table 1 Contents of water, lipid, DNA, RNA and hydroxyproline, mg/g dry wt. in control, sham-operated, bile duct-ligated and thioacetamide-treated male rat livers (– not done)

	H_2O	Lipid	DNA	RNA	Hydroxyproline
Ligated					
Sham-op.					
2 wk	3101 ± 109[a]	353 ± 26[a]	3.17	31.6	1.19 ± 0.08[a]
6 wk	2868 ± 35[a]	391 ± 44[a]	1.97	30.5	1.15 ± 0.05[a]
Test					
2 wk A	3732 ± 12[a]	430 ± 10[a]	26.3	21.4	2.03 ± 0.04[a]
2 wk B	3716	451	–	–	3.68
4 wk B	4096 ± 207[a]	464 ± 118[a]	20.4	35.4	2.75 ± 0.78[a]
6 wk A	4771 ± 161[a]	502 ± 17[a]	22.2	36.2	5.46 ± 0.16[a]
6 wk B	5180 ± 286[a]	531 ± 75[a]	27.7	31.3	8.09 ± 0.96[a]
8 wk A	5105 ± 65[a]	463 ± 58[a]	28.4	36.2	9.66 ± 1.68[a]
8 wk B	4247 ± 110[a]	491 ± 41[a]	15.1	35.1	4.82 ± 1.03[a]
8 wk C	3821	408	21.6	34.9	6.15
Thioacetamide					
Control					
4 wk	2938 ± 13[a]	344 ± 20[a]	6.0	32.0	0.88 ± 0.12[a]
12 wk	3002 ± 29[a]	397 ± 15[a]	4.9	26.1	0.97 ± 0.34[a]
Test					
4 wk (2 livers)	4214 ± 200[a]	1210 ± 148[a]	3.5	15.1	3.01 ± 0.58[a]
8 wk A	4103	509	–	–	2.39
8 wk B	4127 ± 2[a]	517 ± 67[a]	15.9	22.6	2.59 ± 0.53[a]
12 wk A	4461	579	–	–	5.35
12 wk B	4666 ± 66[a]	671 ± 25[a]	23.6	17.6	3.25 ± 0.04[a]

[a] Determinations on two separate aliquots of the same liver

N-desulphated, *N*-acetylated heparin was 42% ($n = 10$) recovered.

Collagen contents (mg/g dry wt.) increased 6- to 10-fold in the ligated livers (0.9→9.0), and ~3-fold in thioacetamide-treated livers (0.9→2.5) (Table 1).

Hyaluronan (nmol hexosamine/g dry wt) increased greatly in ligated livers, and less dramatically in thioacetamide-treated livers (Table 2). HA increased markedly in human cirrhotic liver (Murata et al. 1985), although, as in our experiments, it remained a minor component.

Chondroitin sulphate (=chondroitinase AC chromogen minus hyaluronan lyase chromogen) (nmol hexosamine/g dry wt.) increased strongly in ligated livers (55→1000) and less so in thioacetamide-treated livers (60→200) (Table 2), but remained approximately constant as a ratio to collagen content, in both groups of livers. Probably much of this material was part of DS polymers, since DS ran more quickly on electrophoresis after chondroitinase AC digestion, suggesting that their mol. mass was reduced by the lyase. CS was a minor component, as in human cirrhotic liver (Murata et al. 1985).

Dermatan sulphate (=difference between chondroitinase ABC and AC chromogens) (nmol hexosamine/g dry wt) increased markedly in ligated livers (130→2000) and less strongly in thioacetamide-treated livers (73→1260) (Table 2). The ratio to collagen increased 2- to 3-fold (Fig. 4). Electrophoresis in 0.1 M HCl showed that most DS was normally (~1.0 per disaccharide unit) sulphated. Control liver DS contained a faster moving component, presumably oversulphated, and the proportion of this material to "normal" DS decreased markedly in late post-ligation samples, and to a lesser extent in thioacetamide-treated liver DS. Most of the increase in DS occurred in the "normal" DS, rather than in the oversulphated material, confirming the findings of Suzuki et al. (1976).

CECs of Alcian-blue-complexed normal DS (>0.5 M $MgCl_2$) were significantly higher than those of HS (see below), as were Cupromeronic blue CECs (>0.3 M $MgCl_2$), implying that DS was more highly sulphated than HS. This is compatible with the higher electrophoretic mobility of DS in 0.1 M HCl (see below).

Heparan sulphate was calculated:

1. As nmols 2-sulphamato glucose, based on a heparin standard, which had a hexosamine content of 0.97 μmol/mg; a repeating unit molecular mass of 597 and a sulphate: carboxylate ratio of 2.2:1, by cetylpyridinium titration (Scott 1960). It was assumed that the deamination products from the standard and test molecules gave similar fluorescence yields. Heparin gave

Table 2 Contents of hyaluronan, chondroitin sulphate[a], dermatan sulphate and heparan sulphate, nmol hexosamine/g dry wt., in control, sham-operated, bile duct ligated and thioacetamide-treated rat livers (– not done)

	Hyaluronan	Chondroitin[a] sulphate	Dermatan sulphate	Heparan sulphate	
				N-Ac	N-SO_3
Ligated					
Sham op					
2 wk	12	55	113	178	180
6 wk	10	54	145	170	166
Test					
2 wk A	108 ± 27[b]	162	624	802	509 ± 101[b]
2 wk B	128	–	253	524	408
4 wk B	157	172	471 ± 99[b]	1029 ± 145[b]	569 ± 73[b]
6 wk A	588	544	1149	2707	1155 ± 97[b]
6 wk B	329 ± 51[b]	839	1175	2268	1215 ± 67[b]
8 wk A	1480	995	2178 ± 98[b]	2831 ± 24[b]	1592 ± 44[b]
8 wk B	103 ± 10[b]	249 ± 42[b]	718 ± 198[b]	1012 ± 14[b]	826 ± 17[b]
8 wk C	267	183	713	1216	941
Thioacetamide					
Control					
4 wk	26	51	81	181	232
12 wk	27	71	66	172	242
Test					
4 wk (2 livers)	29 ± 2[b]	65 ± 9[b]	285 ± 27[b]	181 ± 4[b]	242 ± 9[b]
8 wk A	–	–	253	364	175
8 wk B	83 ± 59[b]	76 ± 56[b]	316 ± 21[b]	482 ± 53[b]	223 ± 19[b]
12 wk A	65	110	1250	706	394
12 wk B	62	191	387	542	350 ± 53[b]

[a] Defined as chondroitin lyase AC-produced thiobarbiturate chromogen
[b] Two separate aliquots of liver were analysed

about 70% of the fluorescence of glucosamine itself (Scott 1979), and HS would be expected to lie somewhere between the two.

2. As nmol 2-acetamido glucose, based on the *N*-desulphated, *N*-acetylated heparin standard, which had a repeating unit molecular mass of 418 by cetylpyridinium titration and a hexosamine content of 0.95 μmol/mg. It did not contain sulphamato-glucose (Scott 1979), and on a wt. basis gave about the same amount in thiobarbiturate chromogen as chondroitin-4-sulphate, after digestion with heparinase III or chondroitinase ABC, respectively.

Electrophoresis in 0.1 M HCl showed HS was very heterogeneous. Most was undersulphated (Fig. 1). The sulphate:carboxylate ratio of chondroitinase ABC resistant GAG from L6 by dimethyl-octadecylammonium titration was 0.83. CECs of Alcian blue (0.3–0.5 M $MgCl_2$) or Cupromeronic blue stained HS (<0.2 M $MgCl_2$) were considerably lower than those of the DS, again implying that sulphation was relatively low.

HS as heparinase III chromogen or sulphamato-glucose fluorogen, increased >10-fold, progressively during 8 weeks post-ligation (Table 2). The ratio of the two assays increased from about 1.0 ($n=4$) in the controls to 2.0 in the later stages of fibrosis. The rise in HS in thioacetamide-treated livers was smaller, being hardly significant up to 8 weeks, and only 2- to 3-fold at 12 weeks. Suzuki et al. (1976) observed a 2.3-fold increase in low-sulphated HS in carbon tetrachloride treated liver, similar to our thioacetamide results, but a much larger increase (7-fold) in oversulphated HS, which we identified in small amounts only in normal rat liver. From control to late stage samples higher-sulphated HS declined (Fig. 2) – although in carbon tetrachloride-induced fibrosis (Suzuki et al. 1976) it increased.

There was a small rise in the HS:collagen ratios in both animal models. The ratio to DNA fell in the 4 weeks post-ligation, then recovered. In the thioacetamide-treated livers this ratio fell but did not recover.

Ultrastructure

Alcian blue CEC

At 0.2 M $MgCl_2$ there was little staining in control and early experimental livers, except in blood vessels, the capsule and occasional mast cells. A very faint meshwork of pericellular dimensions was visible. After more than 4 weeks post-ligation, or 8 weeks on thioacetamide, staining at <0.6 M $MgCl_2$ increased considerably, particularly around the blood vessels, and the number of mast cells was greater (results not shown).

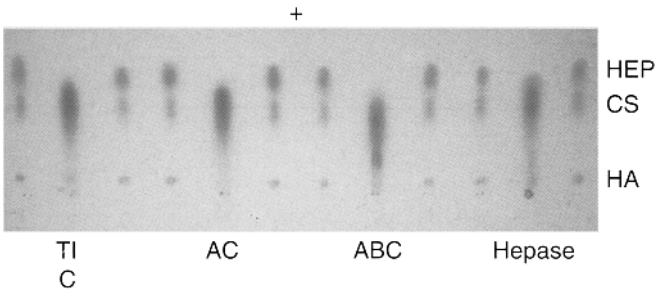

Fig. 1 Electrophoretic patterns in 0.1 M HCl at 2 v/cm, on cellulose acetate membranes of AGAGs from rat liver, stained with Alcian blue. In this system AGAGs run more rapidly the higher the degree of sulphation (Wessler 1970). Standard HA, CSA and heparin were run in parallel. The strips were 5 cm long by 9 cm wide, loaded with 3–6 μg AGAG. AGAGs from T1C control animals, treated with chondroitinase AC (AC), chondroitinase ABC (ABC) and heparinase III (Hepase), respectively. T1C is undigested total AGAG from T1C liver. Most of the highly sulphated material, running in the CSA/heparin region, is removed by chondroitinase ABC digestion. Much of this material is not digested by chondroitinase AC. It is therefore DS. The less highly sulphated material, running between the HA and CS positions, removed by heparinase III digestion but not by chondroitinase ABC is therefore undersulphated HS. This material stained with Cupromeronic blue at CECs <0.2 M $MgCl_2$, in contrast to the DS, which stained at >0.3 M $MgCl_2$. The oversulphated AGAG in the DS region decreased in ligated liver samples, and to a lesser extent in the thioacetamide-treated liver GAGs. The higher-sulphated HS decreased with time in both ligated and thioacetamide-treated liver AGAGs

Electron microscopy and Cupromeronic blue CEC staining

In control and early test livers connective tissue was sparse. Normal collagen fibril morphology was observed, in close-packed parallel bundles, with normal a–e banding patterns which were oriented parallel and antiparallel to those on neighbouring fibrils with about equal frequencies. Most fibril diameters were in the range 30–55 nm (Fig. 2).

In late experimental samples connective tissue was more abundant. Although individual collagen fibrils appeared normal, they were often hapazardly arranged. a–e banding patterns were normal, arranged parallel or antiparallel on neighbouring fibrils with about equal frequencies. Most fibril diameters were in the range 20–35 nm (Fig. 2).

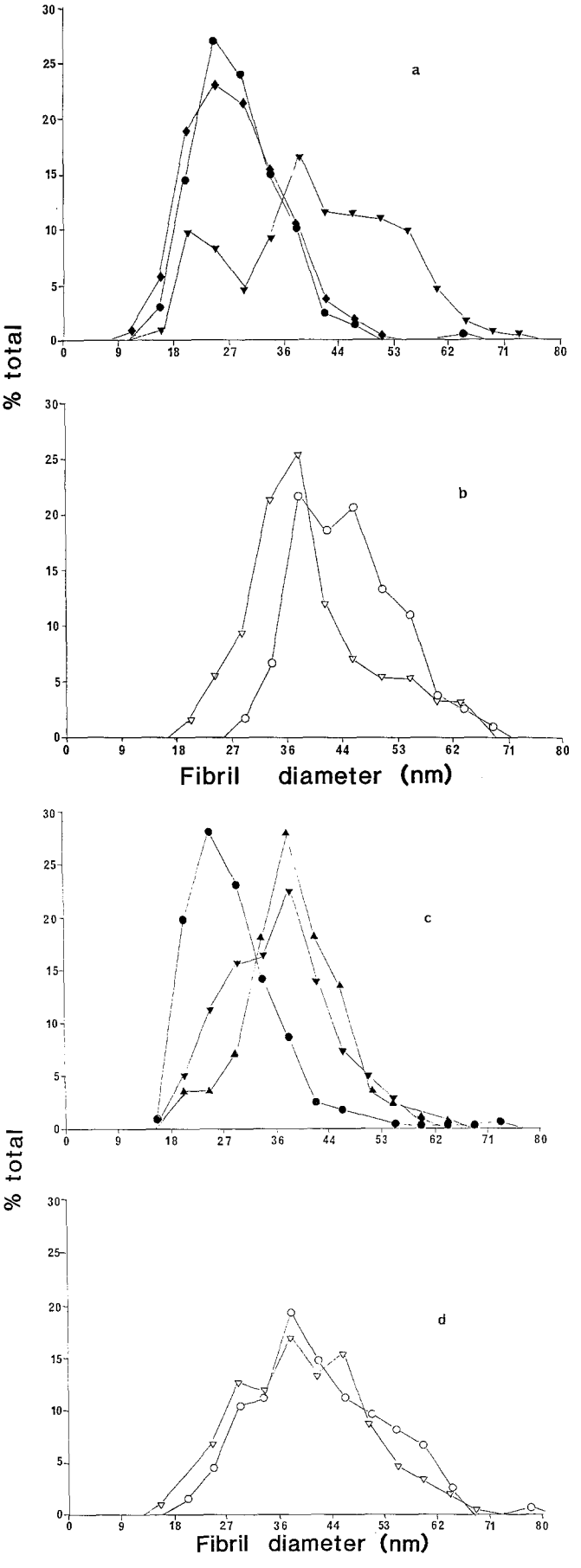

Fig. 2 Collagen fibril diameters from connective tissue in ligated liver (**a, b**) and thioacetamide-treated liver (**c, d**). *Closed symbols* refer to fibrotic tissues, *open symbols* to control livers. ▼L2, ●L6, ◆L8, ▽LC2, ○LC6. ▲T1, ▼T2, ●T3, ▽T1C, ○T3C. The data are plotted as % of total number of fibrils from the relevant tissue (e.g. L2). Total numbers were L2,211; L6,254; L8,457; LC2,126; LC6,120; T1,179; T2,111; T3,361; T1C,268; T3C,135. Fibril diameters decreased with time after the onset of fibrosis. The broad distribution in control tissues narrowed with time, and hints of discrete populations having characteristic fibril diameter distributions within the overall distribution are evident in e.g. L2 and T1. In contrast to the control tissues, the percentage of large fibrils of >53 nm in the latter fibrotic tissues is very small

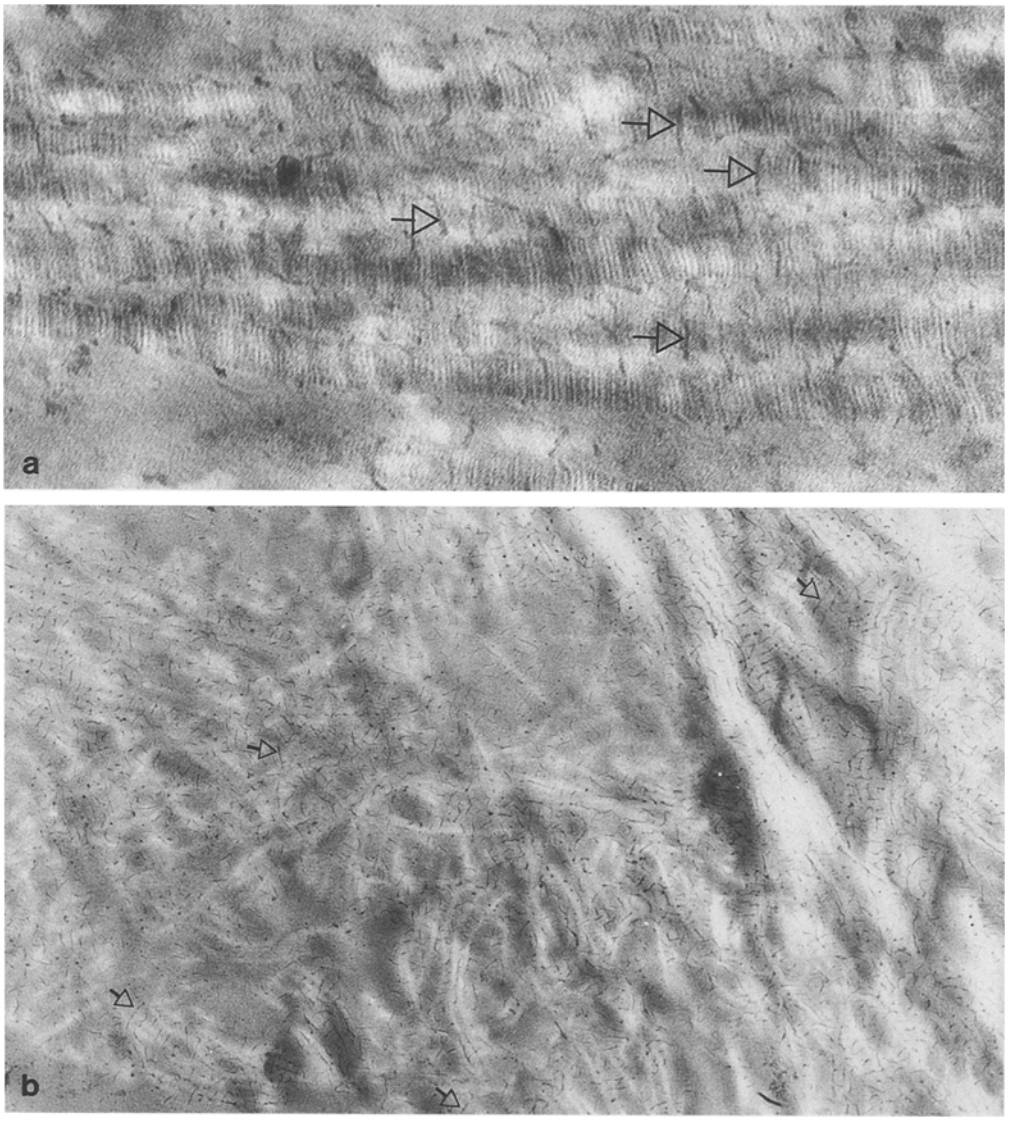

Fig. 3 Electron micrographs of rat liver connective tissue: **a** normal, **b** fibrotic (L8). **a** Cupromeronic blue stained PG filaments are arrowed. Countertained with uranyl acetate to show the collagen fibril banding pattern. The fibrils are ordered, resembling the arrangement in e.g. tendon; some are antiparallel in their banding pattern polarity (× 110000). **b** A frequently seen picture of entangled collagen fibrils, unstained, showing as light lines against the darker plastic. PG filaments are regularly aligned along the fibrils (× 28000)

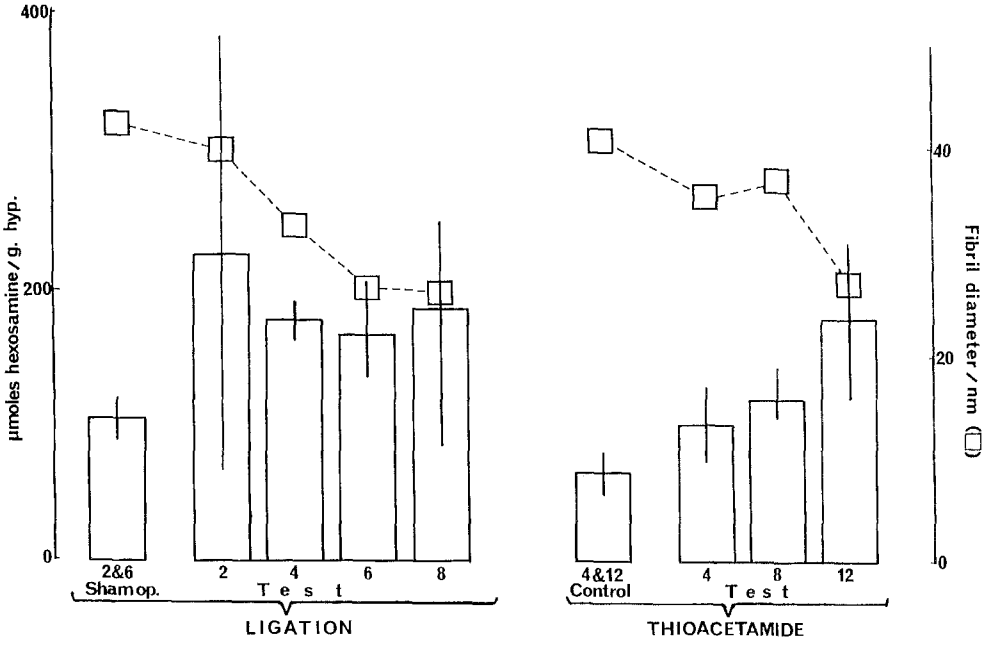

Fig. 4 Histograms of ratios of DS/collagen (hyp) contents of control and fibrotic livers, together with plots of collagen fibril diameters (□). Column heights are averages of measurements on that group of tissues. The *vertical bars* indicate the range of values, each in duplicate, of samples taken from the livers of two or more animals. Collagen fibril diameters are plotted as means of their populations. DS/Hyp ratios increased with time in both groups of livers, as collagen fibril diameters decreased. This is as expected from the finding that PDSs are regularly present on the surface of the fibrils (see text for discussion)

Cupromeronic blue in 0.3 M $MgCl_2$ stains polyanions with degree of sulphation >0.6–0.8 per disaccharide unit. PGs thus stained were regularly and orthogonally arrayed across collagen fibrils at the d & e bands, in normal, early and late experimental samples, These filaments often bridged from one fibril to another (Fig. 3), as in young tendon (Scott 1988). Some were arrayed parallel to the axis of the fibrils. All fibril-associated PG filaments were removed by chondroitinase ABC digestion, but many still remained after chondroitinase AC digestion, strongly suggesting that they contained DS. Lengths (~38 nm) and widths of stained PG filaments were similar in normal and experimental samples.

The ratio of d to e band occupancies changed little throughout the experiment, being about the same as in normal liver (0.6–1.2). Statistics for normal tissue were less numerous than for fibrotic tissue.

Needle-like filaments of stained material were seen in tissue adjoining the bundles of connective tissue. They were not removed completely by chondroitinase ABC, or nitrous acid before staining. They were too few to be unequivocally categorised.

Sex differences

There were no statistically significant differences between male and female rats.

Discussion

Our results mainly relate to *new* tissue, laid down during the experiment. Clinical fibrosis often develops over a much longer time scale allowing remodelling in abnormal environments. Comparisons with human material will not be made.

The two models of fibrosis induction differed markedly in their rates of biochemical and ultrastructural change. Ligation produced much more connective tissue, earlier. Twelve-week thioacetamide-treated livers were not as fibrotic as 8-week-ligated livers. Only increases in fat content were comparable. Trends sometimes moved in opposite directions (e.g. HS/DNA). Fibrosis thus appears as the product of different processes, working at different relative rates in the two models.

Inter-animal varation was particularly marked in the late ligation samples, from animals close to maximum survival time. It seems unwise to interpret results in detail after more than 6 weeks post ligation.

Our biochemical findings extend those previously reported (Suzuki et al. 1976) on rat livers. Big increases in collagen, HA, CS, DS, and HS reflected the increased amounts of connective tissue observed by light and electron microscopy. Concentrations were higher than previously reported, because we took account of recoveries, using internal standards. The dialysis step of Suzuki et al. (1976) led to appreciable (~40%) losses in our hands, although the efficiencies of the two proteolytic digestions (papain vs pronase) were much the same (unpublished work).

A part of the observed increases in duct-ligated liver AGAGs may have been due to blockage of normal catabolism and/or excretion via the bile. Liver HA changed little in thioacetamide-treated animals, compared with dramatic increases post-ligation, although considerable amounts of new connective tissue were laid down in both. HA is cleared via the liver (Laurent and Fraser 1992). Similarly, the dramatic rise in DNA content in ligated, compared with thioacetamide-treated livers, may also reflect accumulation from the blood of macromolecular DNA (Emlen and Mannik 1978), which is then not broken down and/or excreted via the bile. However, the marked increase in the number of nuclei, particularly evident in ligated livers, probably accounts for much of the increase in DNA.

HS, which was a major AGAG, increased as dramatically as DS and HA. HS/DNA fell during fibrosis induction, which is unexpected if the latter were a simple measure of total cellularity and HS were cell-bound, but is explicable on the basis that some cells were multinucleate, and that different cell types may not have the same HS/DNA ratios.

Our finding that isolated HS was mainly undersulphated, relative to DS, does not accord with claims that some rat liver HS was highly (1.4) sulphated (Suzuki et al. 1976). Their reported electrophoretic mobilities are compatible with our data, which do not suggest high sulphation (Results). Recoveries of HA, CS and heparin were 60–100%, implying we had not lost all high-sulphated HS – and our findings on high-sulphated DS compare well with those of Suzuki et al. (1976). Higher-sulphated HS declined relative to other AGAGs as fibrosis increased (Results); an opposite trend to that reported previously (Suzuki et al. 1976), but parallelling the decrease in DS sulphation (above). Carbon tetrachloride may effect HS in fibrosis differently, compared with thioacetamide administration or bile duct ligation.

Sulphamato-glucose:acetamido-glucose ratios resembled those previously reported (Suzuki et al. 1976) based on assays of acid labile and total ester sulphate. They varied markedly with the severity of fibrosis (1.0→0.4, Table 3), suggesting that biosynthesis of HS was specifically effected at the *N*-sulphation stage, in which the *N*-acetyl is replaced. The overall decrease in sulphation of DS and HS (Results) may reflect a diminution in PAPS supply, perhaps due to the increased demands in detoxifying accumulated metabolites.

We did not observe HS associated with collagen fibrils on staining with Cupromeronic blue at 0.1 M $MgCl_2$, at which CEC our isolated HS was stained, all fibril-associated sulphated AGAG was removed by chondroitinase ABC digestion. It appears that HS is not part of the collagenous fibrillar structures although the increase in HS paralleled the increase in collagen. HS is said to be associated mainly with basement membranes and cell surfaces (Gallagher et al. 1986).

How "good" was the new connective tissue?

Qualitatively, the criteria indicate that it was "normal", closely resembling, for example, young tendon. Thus, the new collagen fibrils were of a fairly constant diameter (~25 nm), with a normal banding pattern (a–e), as in young tendon. Fibril alignments with parallel and antiparallel a–e banding patterns were roughly equal, as in normal connective tissues (Scott and Parry 1992). Sulphated PGs were present at d & e bands (Fig. 3), but not elsewhere along the fibril, as expected given that the AGAGs were DS-rich (Scott 1988). This was true of both the high-sulphated AGAGs (staining at 0.3 M $MgCl_2$) and low-sulphated PGs (staining at 0.1 M $MgCl_2$, Results).

The ratio of d/e band occupancy varies from tissue to tissue. In mature rat tail tendon it was as high as 5 (Scott and Haigh 1985), and in bovine corneal stroma, as low as 1.3 (Scott and Haigh 1987). In normal and fibrotic liver it was even lower; 0.6–1.2 (Results). The oversulphated structure (2-acetamido, 4,6 galactose bisulphate) in rat liver differed from that in skin (Suzuki et al. 1976). Since the PDS at the e band was more highly sulphated than that at the d band in corneal stroma (Scott and Haigh 1988), a low d/e band occupancy ratio might imply a high average sulphation, which is compatible with the finding of oversulphated DS in normal rat liver (Results). However, although the oversulphated DS declined in proportion to normal DS in fibrotic rat liver (Results), d/e band occupancies changed little. Better statistics of band occupancies in control tissues are required. Our findings on DS qualitatively confirmed those (Suzuki et a. 1976) in fibrosis induced by administration of carbon tetrachloride.

Quantitatively, too, our findings suggest that the fibrotic tissue is "normal". PDS is associated with collagen, and the rise in total collagen accompanied a rise in total DS (Results; Scott 1988). The ratio of DS to total collagen depends on the surface area of the fibrils, since PDSs are located at the fibril surface, regularly and specifically. Thus, a given amount of collagen, distributed in fibrils of diameter r, has twice the surface area if distributed in fibrils of diameter $0.5\,r$, and hence twice the ratio of DS to collagen (Scott 1984). This is compatible with what we found; a decrease in the average fibril diameter accompanied a roughly two-fold increase in the ratio of DS to collagen in the fibrotic tissue (Fig. 4).

Lengths and thicknesses of Cupromeronic-blue-stained PG filaments were similar in tests and controls (Results), suggesting that AGAG duplexes and higher aggregates (Scott 1992) were similar structures in each case. This length (~38 nm) corresponds to a M_r of about 20 kDa for the DS chain, which is similar to that of skin and sclera DS, and considerably less than that of corneal stroma DS (Scott 1992).

Thinner fibrils (~25 nm) were associated with higher levels of CS and HA (Fig. 2, Table 2) than were found in (normal liver) tissues with thicker fibrils, as was also the case in three different tendons from three different species (Scott 1986).

Thus, at a high level of supramolecular organisation, the new connective tissue resembled other developing connective tissues. Fibrils tended to be disorganised (Fig. 3), unlike the well-ordered parallel sheets and bundles found in tendons, skin, sclera, cornea etc. The new tissue was laid down in the midst of already organised tissue, and the absence of a suitable template probably produced tangled groups of fibrils.

In summary, we find that the new ECM laid down in experimental liver fibrosis is "good", according to the guidelines on supramolecular organisation established on normal ECM. It resembles new ECM produced during normal development of, for example, young tendon. It is deposited in the wrong place.

References

Bitter T, Muir HM (1964) A modified uronic acid carbazole reaction. Anal Biochem 4:330–334

Emlen W, Mannik M (1978) Kinetics and mechanisms for removal of circulating single-stranded DNA in mice. J Exp Med 147:684–699

Gallagher JT, Lyon M, Steward WP (1986) Structure and function of heparan sulphate proteoglycans. Biochem J 236:313–325

Gressner AM (1991) Liver fibrosis: perspectives in pathobiochemical research and clinical outlook. Eur J Clin Biochem 29:293–311

Gressner AM, Pazen H, Greiling H (1977) The synthesis of total and specific glycosaminoglycans during development of experimental liver cirrhosis. Experientia 1977, 33:1290–1292

Haigh M, Scott JE (1986) A method of processing tissue sections for staining with Cupromeronic blue and other dyes, using CEC techniques, for light and electron microscopy. Bas Appl Histochem 30:479–486

Kissane JM, Robins E (1958) The fluorimetric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. J Biol Chem 233:184–188

Kountouras J, Billing BH, Scheur PJ (1984) Prolonged bile duct obstruction: a new experimental model for cirrhosis in the rat. Br J Exp Pathol 65:305–311

Laurent TC, Fraser JR (1992) Hyaluronan. FASEB J 6:2397–2404

Muller A, Machnik F, Zimmermann T, Schubert H (1988) Thioacetamide-induced cirrhosis-like liver lesions in rats – usefulness and reliability of this animal model. Exp Pathol 34:229–236

Murata K, Ochiai Y, Akashio K (1985) Polydispersity of acidic glycosaminoglycan components in human liver and the changes at different stages in liver cirrhosis. Gastroenterology 89:1248–1257

Scott JE (1960) Aliphatic ammonium salts in the assay of acidic polysaccharides from tissues. Methods Biochem Anal 8:145–197

Scott JE (1979) A reaction for the simple sensitive fluorimetric assay of heparin and 2-amino sugars. Biochem J 183:91–97

Scott JE (1984) The periphery of the developing collagen fibril. Quantitative relationships with dermatan sulphate and other surface-associated species. Biochem J 218:229–233

Scott JE (1986) Proteoglycan-collagen interactions. Ciba Found Symp 124:104-124

Scott JE (1988) Proteoglycan-fibrillar collagen interactions. Biochem J 252:313–323

Scott JE (1992a) Supramolecular organisation of extracellular matrix glycosaminoglycans, in vitro and in the tissues. FASEB J 6:2639–2645

Scott JE (1992 b) Morphometry of Cupromeronic blue-stained proteoglycan molecules in animal corneas, versus that of purified proteoglycans stained in vitro, implies that tertiary structures contribute to corneal ultrastructure. J Anat 180:155–164

Scott JE, Bosworth TR (1990) A comparative biochemical and ultrastructural study of proteoglycan-collagen interactions in corneal stroma. Biochem J 270:491–497

Scott JE, Dorling J (1965) Differential staining of acid glycosaminoglycan (mucopolysaccharides) by Alcian blue in salt solutions. Histochemie 5:221–233

Scott JE, Haigh M (1985) "Small" proteoglycan: collagen interactions: keratan sulphate proteoglycan associates with rabbit corneal collagen fibrils at the a' and c' bands. Biosci Rep 5:765–774

Scott JE, Haigh M (1988) Identification of specific binding sites for keratan sulphate proteoglycans and chondroitin-dermatan sulphate proteoglycans on collagen fibrils in cornea by the use of Cupromeronic blue in critical electrolyte concentration' techniques. Biochem J 253:607–610

Scott JE, Parry DAD (1992) The control of collagen fibril diameters in connective tissues. Int J Biol Macromol 14:292–293

Scott JE, Tigwell MJ (1978) Periodate oxidation and the shapes of glycosaminoglycuronans in solution. Biochem J 173:103–114

Suzuki S, Suzuki S, Nakamura N, Koizumi T (1976) The heterogeneity of dermatan sulfate and heparan sulfate in rat liver and a shift in the glycosaminoglycan contents in carbon tetrachloride-damaged liver. Biochim Biophys Acta 428:166–181

Trams EG, Symeonidis A (1957) Morphological and functional changes in the livers of rats after ligation and excision of the common bile duct. Am J Pathol 33:13–27

Wessler E (1970) Electrophoretic separation of glycosaminoglyans excreted in excess in different kinds of "mucopolysaccharidoses." Academic Press, London, New York. In: Balazs EA (ed) Chemistry and molecular biology of the extra-cellular matrix, vol 2. pp 895–901

Woessner JF Jr (1961) Determination of hydroxyproline in tissues and protein samples containing small proportions of this imino acid. Arch Biochem Biophys 93:440–447

Zimmermann T, Muller A, Machnik G, Franke H, Schubert H, Dargel R (1987) Biochemical and morphological studies on production and regression of experimental liver cirrhosis induced by thioacetamide in Uje: Wist rats Z Versuchstierkd 30:165–180