Biochem. J. (1985) **228**, 443–450
Printed in Great Britain

Absence of keratan sulphate from skeletal tissues of mouse and rat

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(*Received 29 November 1984/7 February 1985; accepted 22 February 1985*)

The absence of keratan sulphate synthesis from skeletal tissues of young and mature mice and rats has been confirmed by (1) analysis of specific enzyme degradation products of newly synthesized glycosaminoglycans, and (2) immunohistochemistry and radioimmunoassay using a monoclonal antibody directed against keratan sulphate. Approx. 98% of the [^{35}S]glycosaminoglycans synthesized *in vivo* by mouse and rat costal cartilage, and all of those of lumbar disc, are chondroitin sulphate. The remainder in costal cartilage were identified as heparan sulphate in mature rats. In contrast, [^{35}S]glycosaminoglycans synthesized by cornea of both species comprised both chondroitin sulphate and keratan sulphate. In mice keratan sulphate accounted for 12–25% and in rats 40–50% of the total [^{35}S]glycosaminoglycans, depending on the age of the animal. Experiments *in vitro* with organ culture of cartilage and cornea confirm these results. Absence of keratan sulphate from mouse costal cartilage and lumbar disc D1-proteoglycans was corroborated by inhibition radioimmunoassay with the monoclonal antibody MZ15 and by lack of staining for keratan sulphate in indirect immunofluorescence studies using the same antibody.

Proteoglycans are a major macromolecular component of connective tissue extracellular matrix. In addition to their classical role of contributing to viscoelasticity (Kempson *et al.*, 1976), they may have important roles in controlling the development and maturation of skeletal and other tissues (Hascall & Hascall, 1981; Toole, 1981).

Cartilage contains heterogeneous populations of aggregating and non-aggregating large proteoglycans relatively enriched in chondroitin sulphate, or keratan sulphate, and also small non-aggregating proteoglycans (Heinegård *et al.*, 1981, 1985). In addition to glycosaminoglycan chains, proteoglycan core protein also bears *O*-linked (Lohmander *et al.*, 1980) and *N*-linked (De Luca *et al.*, 1980) oligosaccharides.

Intervertebral disc proteoglycans are thought to be of similar structure to the chondroitin sulphate–keratan sulphate proteoglycans of hyaline cartilage (Stevens *et al.*, 1979). Oligosaccharides have yet to be identified on intervertebral disc proteoglycans.

The composition of bovine and human cartilage proteoglycans changes during maturation from predominantly chondroitin sulphate-rich molecules in early life to predominantly keratan sulphate-rich molecules in mature and ageing tissues (Roughley & White, 1980; Sweet *et al.*, 1979). The increase in keratan sulphate content may occur by increased substitution of *O*-linked oligosaccharides with keratan sulphate chains (Garg & Swann, 1981; Sweet *et al.*, 1979; Roughley *et al.*, 1981). Similar increases in keratan sulphate content with age have been noted for intervertebral disc proteoglycans (Adams & Muir, 1976; Ghosh *et al.*, 1977). It seems likely that the keratan sulphate-rich proteoglycans arise not, as previously thought, by cleavage of the chondroitin sulphate-rich region of core protein (Sweet *et al.*, 1979; Bayliss *et al.*, 1984), but from a switching in chondrocytes to synthesis of this type of molecule.

Keratan sulphate is not present in either intervertebral discs or costal cartilage of young CBA mice (Venn & Mason, 1983), or in proteoglycans isolated from the Swarm rat chondrosarcoma (Oegema *et al.*, 1975). We report below that these species do not synthesize skeletal keratan sulphate (KS II), at any stage in maturation, even though corneal keratan sulphate (KS I) is made.

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Materials and methods

Materials

Special reagents were obtained as follows: Sephadex G-50 and Sephadex G-25 (PD10 columns) (Pharmacia), whale and shark cartilage chondroitin sulphate and twice recrystallized papain (Sigma), bovine corneal keratan sulphate, chondroitinase ABC and keratanase (Miles Biochemicals), tissue culture reagents (Gibco Europe), radioisotopes (Amersham International).

Labelling procedures

Male CBA mice or Wistar rats were given an intraperitoneal injection of $Na_2{}^{35}SO_4$ (5mCi/μg of sulphur, carrier-free, 20mCi/kg for mice and 2mCi/kg for rats), the dose was repeated 2h later, and the animals were killed 2h later. Costal cartilage slivers, intervertebral discs, and corneas were removed.

For organ cultures *in vitro* whole corneas and costal cartilage slivers from CBA mice were incubated in 2ml of Dulbecco's modified Eagles medium (sulphate-free) containing 5% (v/v) foetal-calf serum, 100 units of penicillin and streptomycin/ml, 500μCi of $Na_2{}^{35}SO_4$ (5mCi/μg)/ml and 100μCi of D-[6-^{3}H]glucosamine hydrochloride (38Ci/mmol)/ml for 18h at 37°C in an air/CO_2 (19:1) atmosphere. After labelling, media were removed, the scleral rims were trimmed from the cornea, any non-cartilaginous tissues were removed from the costal cartilage, and the tissues were recombined with the media.

Extraction of glycosaminoglycans

Labelled tissues and medium were digested with papain (Venn & Mason, 1983). In some experiments [^{35}S]proteoglycans were extracted with 4.0M-guanidinium chloride/0.05M-sodium acetate, pH5.8, containing proteinase inhibitors (Venn & Mason, 1983). Unincorporated radioactivity was removed from papain digests or 4.0M-guanidinium chloride extracts by chromatography on PD10 columns, eluted with 0.05M-Tris/HCl, pH7.2, in the presence of 1mg of rat chondrosarcoma aA1 proteoglycan (Faltz *et al.*, 1979). In some cases unincorporated radioactivity was removed from papain digests by dialysis (1:100, v/v) three times against distilled water, using a Spectropor membrane with M_r 2000 cut-off.

Preparation of purified mouse proteoglycans

The 4.0M-guanidinium chloride extracts were dialysed (1:100 v/v) three times against 4.0M-guanidinium chloride, pH5.8, containing proteinase inhibitors. D1 proteoglycans were isolated by CsCl density gradient centrifugation using a starting density of 1.43g/ml (Mason *et al.*, 1982). Fractions of density >1.53g/ml were pooled to constitute the D1 fractions. These contained 53% and 64% of the total radioactivity for costal cartilage and lumbar disc respectively.

Analytical procedures

Papain digests containing labelled glycosaminoglycans were digested with either chondroitinase ABC, keratanase or HNO_2 with or without prior treatment with 0.05M-NaOH/1M-$NaBH_4$ (Venn & Mason, 1983). Whale and shark cartilage chondroitin sulphate (chondroitinase digests) or bovine corneal keratan sulphate (keratanase digests) was added to enzyme digests to control for complete digestion. Deamination of labelled glycosaminoglycans by HNO_2 treatment was carried out according to the procedure of Shively & Conrad (1976).

Labelled glycosaminoglycans or digestions thereof were chromatographed on Sephadex G-50 columns (0.6cm × 150cm) eluted at 2.5ml/h with 0.05M-Tris/HCl, pH7.2. Fractions were monitored for hexuronic acid (carbazole assay) or hexose (anthrone assay) by automated procedures (Heinegård, 1973) and for radioactivity.

Immunological studies

The keratan sulphate content of D1 proteoglycan fractions of mouse disc and costal cartilage was determined by inhibition radioimmunoassay using the monoclonal antibody MZ15 raised in mice against an antigenic determinant which is sensitive to keratanase digestion (Zanetti *et al.*, 1985).

Frozen sections of costal cartilage and lumbar disc were incubated with MZ15 and then with fluorescein-conjugated rabbit anti-(mouse IgG) for fluorescent microscopy.

Results and discussion

Synthesis in vivo of mouse skeletal [^{35}S]glycosaminoglycans

[^{35}S]Glycosaminoglycans in papain digests of young and mature mouse cartilage and intervertebral discs were eluted in the void volume of Sephadex G-50 columns (results not shown), but after chondroitinase ABC digestion virtually all the radioactivity moved into two peaks representing chondroitin sulphate disaccharides ($K_{av.}$ 0.93) and linkage region ($K_{av.}$ 0.69) (Figs. 1*a*–1*d*). Keratanase treatment of the whole [^{35}S]glycosaminoglycan fraction did not result in movement of any ^{35}S-labelled material into the included volume of the column, although the carrier bovine corneal keratan sulphate was degraded to di-, tetra- and hexasaccharides (results not shown). The very small amount of material eluted in the void volume of chondroitinase ABC digests of costal cartilage

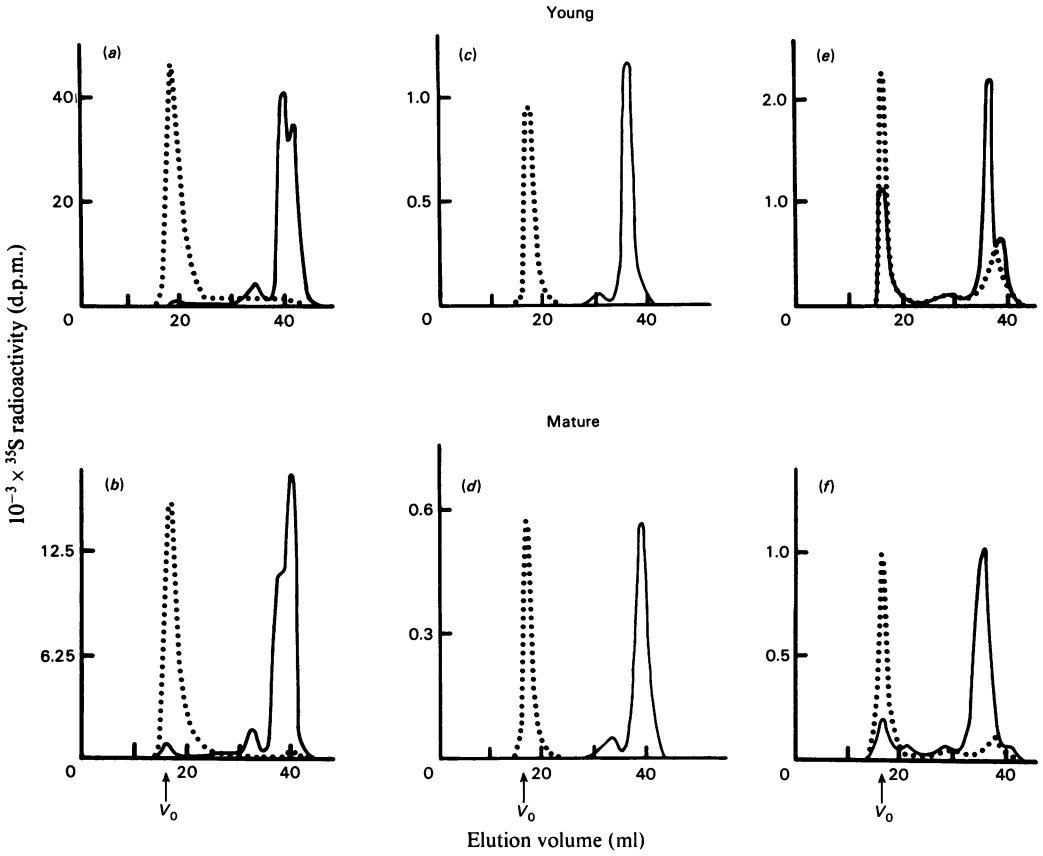

Fig. 1. *Analysis of glycosaminoglycans ^{35}S-labelled in vivo in papain digests of mouse tissues*
Sephadex G-50 chromatography profiles of chondroitinase ABC (——) or keratanase (····) digests of [^{35}S]glycosaminoglycans of 30-day-old (upper panel) and 150-day-old (80-day-old for lumbar disc) (lower panel) mouse costal cartilage (*a*, *b*), lumbar intervertebral disc (*c*, *d*) and cornea (*e*, *f*).

(Figs. 1*a* and 1*b*, solid line) was recovered and found resistant to keratanase treatment. It is probably analogous with similar material found in rat costal cartilage identified as heparan sulphate (see below). Thus virtually all of the newly synthesized [^{35}S]glycosaminoglycans in costal cartilage and lumbar discs of young and mature mice is composed of chondroitin (and/or dermatan) sulphate. Similar results were obtained for thoracic and cervical discs (results not shown).

Synthesis in vivo of mouse corneal [^{35}S]glycosaminoglycans

All [^{35}S]glycosaminoglycans in papain digests of the corneas were eluted in the void volume of Sephadex G-50 chromatography columns (results not shown). Chondroitinase ABC and keratanase digests showed that cornea contained both chondroitin sulphate and keratan sulphate.

The relative amount of keratan sulphate synthesized decreases during ageing from 25% to about 12% of the total glycosaminoglycans, as judged by sensitivity to chondroitinase ABC (Figs. 1*e* and 1*f*, solid line) and resistance to keratanase (Figs. 1*e* and 1*f*, dotted line).

The keratanase digestion products of young mouse cornea were eluted solely as disaccharides (Fig. 1*e*, dotted line), indicating that non-sulphated galactose residues occur in every repeat unit of the chain (Nakazawa & Suzuki, 1975). In older animals, the digestion products are of varying size (Fig. 1*f*, dotted line), indicating that some of the galactose residues are also probably sulphated.

The 'linkage' region peak ($K_{av.}$ 0.69; Figs. 1*a*–1*d*) is not present in chondroitinase ABC digests of corneal glycosaminoglycans, suggesting that these may have a slightly different sequence at the reducing end to those of skeletal tissues. However, there is a small but reproducible peak of ^{35}S radioactivity eluted in the middle of the G-50

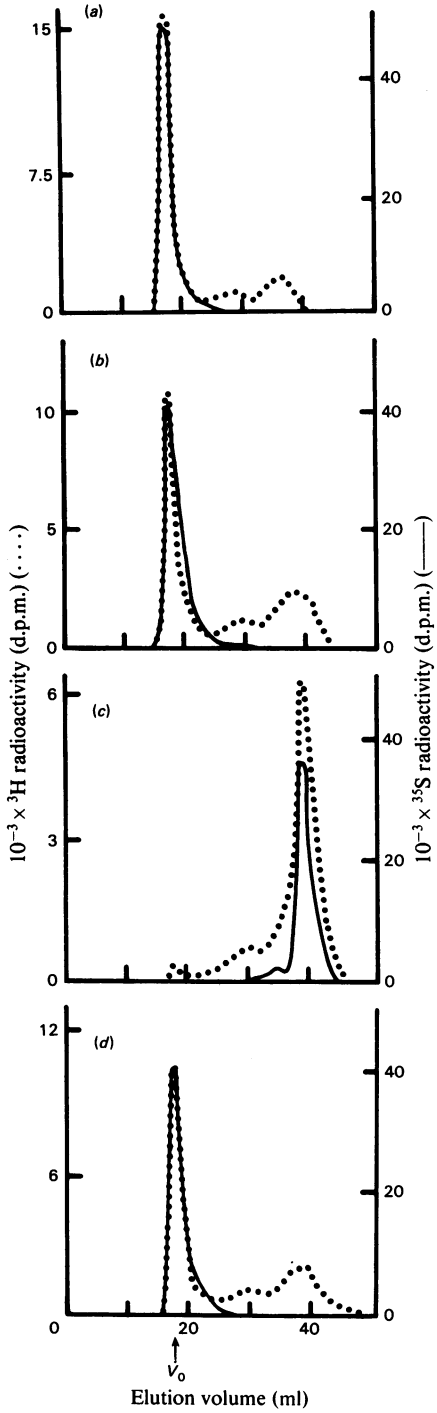

Fig. 2. *Analysis of 30-day-old mouse costal cartilage glycosaminoglycans synthesized during short-term organ culture*

Mouse costal cartilage slivers were incubated for 18h with [^{3}H]glucosamine (····) and $Na_2{}^{35}SO_4$ (——). Papain digested glycosaminoglycans were chromatographed on Sephadex G-50 columns directly (*a*) or after subsequent alkaline borohydride treatment (*b*) or alkaline borohydride and chondroitinase ABC treatment (*c*) or alkaline borohydride and keratanase treatment (*d*).

column run ($K_{av.}$ 0.5) which is not susceptible to chondroitinase ABC (Fig. 1*e*) or keratanase (Fig. 1*f*) treatments. This could be due to a sulphated oligosaccharide.

Synthesis in vitro of mouse skeletal [^{35}S,^{3}H]glycosaminoglycans

The results in Fig. 1 could be due to a lack of sulphation of keratan sulphate in the skeletal tissues *in vivo*. Therefore costal cartilage slivers were incubated with [^{35}S]sulphate and [^{3}H]-glucosamine to enable detection of both non-sulphated and sulphated newly synthesized glycosaminoglycans.

Newly synthesized glycosaminoglycans were eluted in the void volume as shown by the ^{35}S profiles (Figs. 2*a* and 2*b*) and the [^{35}S]glycosaminoglycan enzyme digestion products (Figs. 2*c* and 2*d*) have similar profiles to those obtained *in vivo* (Figs. 1*a* and 1*b*).

All of the ^{35}S-labelled and 97% of the ^{3}H-labelled void volume material is degraded to disaccharides after chondroitinase ABC treatment (Fig. 2*c*) and the amount of ^{3}H-labelled material eluted in the middle of the column run (Fig. 2*a*) is unchanged, indicating that it is not glycosaminoglycan. The small amount of ^{3}H-labelled material left at the void volume after chondroitinase ABC treatment does not appear to be keratan sulphate, as there is no change in the amount of material eluted near the total volume of the column before (Fig. 2*b*) or after (Fig. 2*d*) keratanase treatment. It may represent glycogen. The absence of incorporation of both [^{35}S]sulphate and [^{3}H]glucosamine into keratan sulphate in costal cartilage *in vitro* confirms the result *in vivo* and shows that the latter is not an artefact due to absence of sulphation.

Synthesis in vitro of mouse corneal [^{35}S,^{3}H]-glycosaminoglycans

Mouse corneal glycosaminoglycans labelled with [^{3}H]glucosamine and [^{35}S]sulphate during short-term organ culture were also analysed. Of the [^{35}S]glycosaminoglycans, 90% are sensitive to chondroitinase ABC (Fig. 3*c*, solid line), a finding in agreement with Hassell *et al.* (1979), who showed that corneal stromocytes in culture synthesize a higher proportion of chondroitin sulphate to keratan sulphate compared with the unlabelled molecules in the stroma. Corneal stromocytes in culture may partially lose the capacity to make keratan sulphate proteoglycans (Klintworth & Smith, 1976).

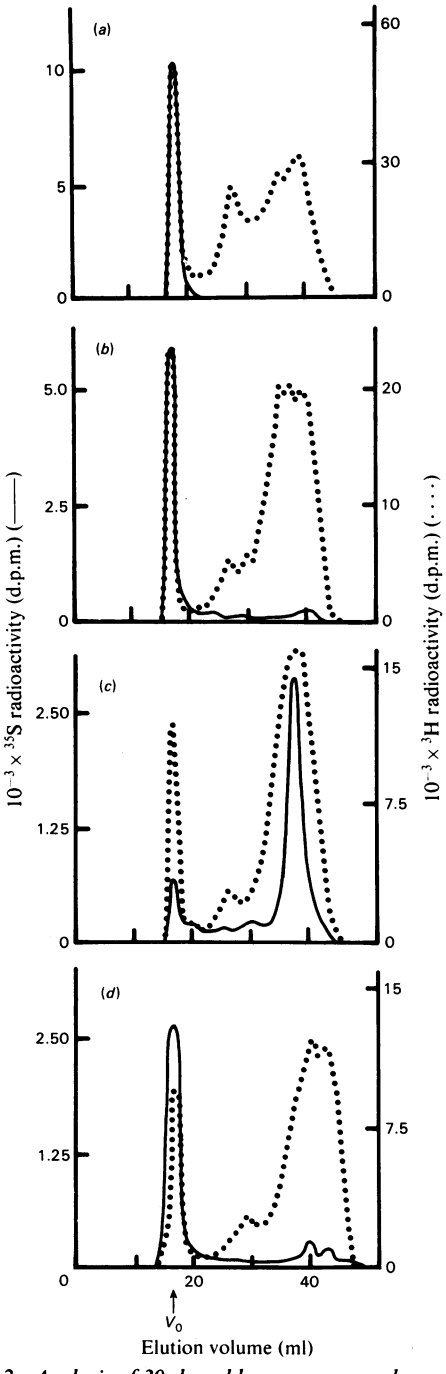

Fig. 3. *Analysis of 30-day-old mouse cornea glycosaminoglycans synthesized during short-term organ culture*
Mouse corneas were incubated for 18h with [^{3}H]glucosamine (····) and $Na_2{}^{35}SO_4$ (——). Papain-digested glycosaminoglycans were chromatographed on Sephadex G-50 columns directly (*a*) or after subsequent alkaline borohydride treatment (*b*), alkaline borohydride and chondroitinase ABC treatment (*c*) or alkaline borohydride/keratanase treatment (*d*).

After papain and alkaline borohydride treatment the void volume ^{3}H-labelled material comprised 15.4% of the total ^{3}H radioactivity (Fig. 3*b*, dotted line). This peak is decreased to 12.5% after chondroitinase ABC treatment (Fig. 3*c*, dotted line) and to 15% after keratanase treatment (Fig. 3*d*, dotted line), indicating that the two [^{3}H]glycosaminoglycans are in the same relative proportions as their ^{35}S-labelled counterparts.

The nature of the remainder of the ^{3}H-labelled void volume material is unknown, but it probably contains some glycogen, and large oligosaccharides from ^{3}H-labelled glycoproteins. It is unlikely to be [^{3}H]hyaluronate, as this is probably digested under the conditions used for chondroitinase treatment (0.0001 unit/μg of glycosaminoglycan, 18h, 37°C).

Synthesis in vivo of rat skeletal [^{35}S]-glycosaminoglycans

All the ^{35}S-labelled material in the papain digests of all tissues was eluted in the void volume fractions of Sephadex G-50 columns (results not shown). After chondroitinase ABC treatment, 97.5% of the [^{35}S]glycosaminoglycans in the papain digest of costal cartilage (Figs. 4*a* and 4*b*, solid lines) and all of those in lumbar discs (Fig. 4*c*, solid line) are degraded to disaccharides and a linkage region peak. Keratanase treatment did not result in movement of ^{35}S-labelled material into the included volume of the column (Figs. 4*a*–4*c*, dotted lines).

To determine the nature of the small amount of material which was not degraded by chondroitinase ABC (Fig. 4*b*), a portion of the papain digest of 250-day-old rat costal cartilage was subjected to HNO_2 treatment prior to chromatography on Sephadex G-50 columns. This resulted in the movement of 2.5% of the ^{35}S-labelled material into the total volume of the column (Fig. 5*a*). A portion of the void volume fraction was recovered and digested with 1 unit of chondroitinase ABC, after which all the ^{35}S-labelled material was eluted in the position of disaccharides and a linkage region peak (Fig. 5*b*). These results demonstrate the presence of small amounts (approx. 2.5% of the total glycosaminoglycans) of heparan sulphate in costal cartilage of mature rats. It is likely that the chondroitinase ABC/keratanase-resistant ^{35}S-labelled glycosaminoglycan in mouse costal cartilage (Figs. 1*a* and 1*b*) is also heparan sulphate.

Synthesis in vivo of rat corneal [^{35}S]-glycosaminoglycans

Approx. 45% of newly synthesized corneal glycosaminoglycans in young rats and 49% of those in old rats are chondroitin sulphate, as judged by

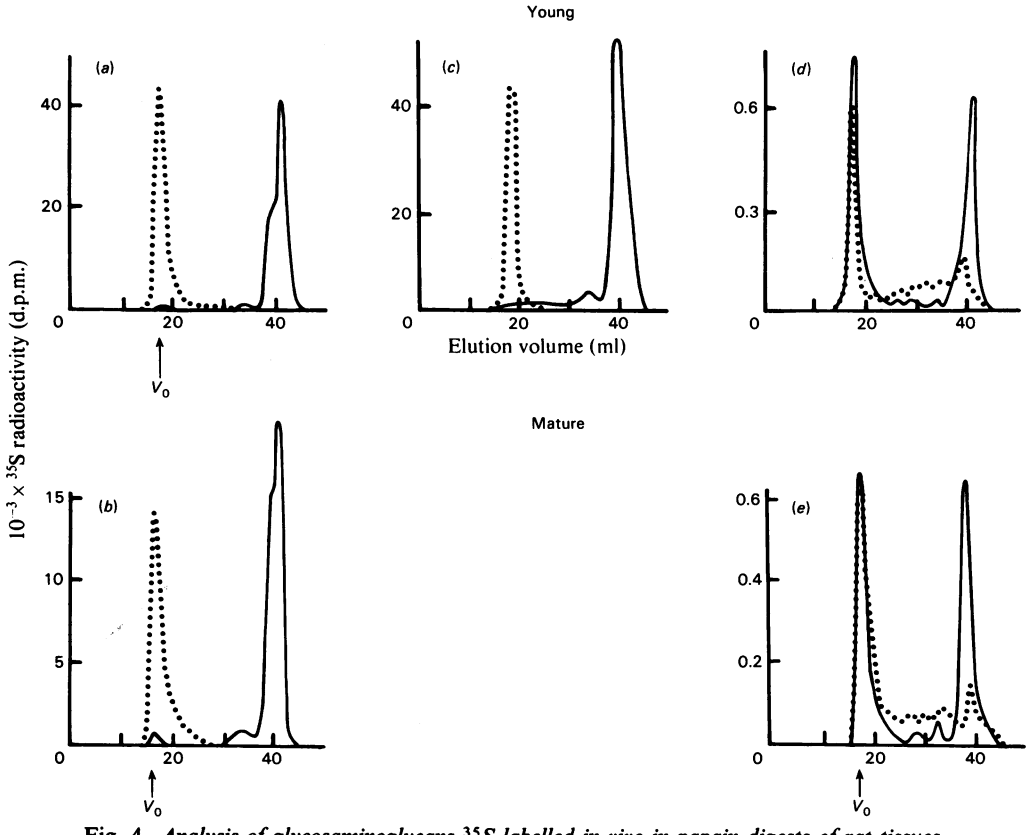

Fig. 4. *Analysis of glycosaminoglycans ^{35}S-labelled in vivo in papain digests of rat tissues*
Sephadex G-50 chromatography profiles of chondroitinase ABC (——) or keratanase (····) digests of [^{35}S]-glycosaminoglycans of 30-day-old (upper panel) and 250-day-old (lower panel) rat costal cartilage (*a*, *b*), lumbar intervertebral disc (*c*) and cornea (*d*, *e*).

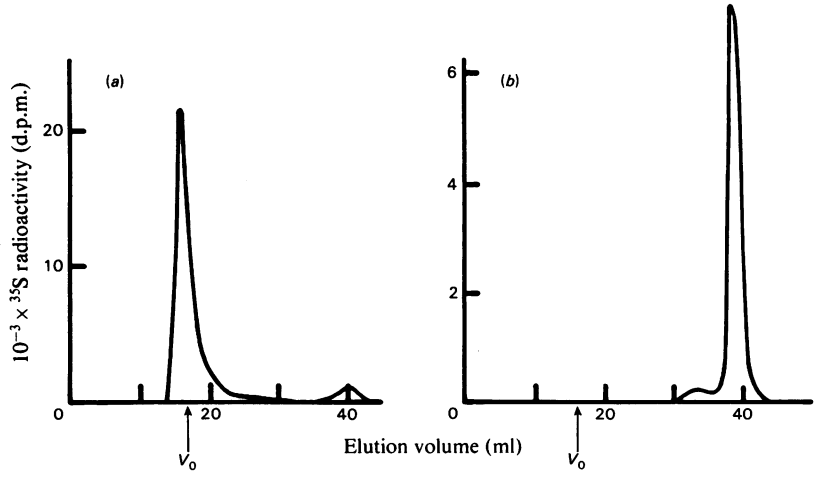

Fig. 5. *Identification of [^{35}S]heparan sulphate in papain digests of rat costal cartilage*
Sephadex G-50 chromatography profiles of (*a*) HNO_2 digest of [^{35}S]glycosaminoglycans of 250-day-old rat costal cartilage, and (*b*) chondroitinase ABC digests of a portion of the V_0 fraction from (*a*).

chondroitinase ABC sensitivity (Figs. 4*d* and 4*e*, solid lines). The remainder are keratan sulphate, as shown by keratanase sensitivity (Figs. 4*d* and 4*e*, dotted lines). Therefore, in contrast with mouse, keratan sulphate is a major glycosaminoglycan in the rat cornea.

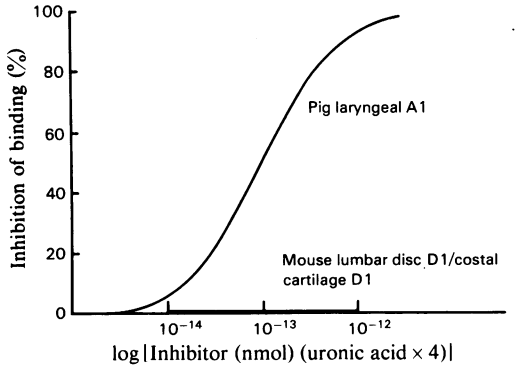

Fig. 6. *Inhibitions of ^{125}I-A1D1 binding to MZ15 by mouse and pig proteoglycans*

Inhibition radioimmunoassay of mouse skeletal proteoglycans

The absence of keratan sulphate in mouse skeletal tissues was confirmed by immunochemical studies using a monoclonal antibody to keratan sulphate, MZ15 (Zanetti *et al.*, 1985). Fig. 6 shows the ability of pig laryngeal A1 and mouse skeletal D1 proteoglycans to inhibit the binding of pig laryngeal cartilage ^{125}I-labelled A1D1-proteoglycan to MZ15. The former contain keratan sulphate and inhibit binding in a dose-dependent manner; however, mouse disc and costal cartilage D1 proteoglycans are completely unable to inhibit this binding, showing that they do not contain the epitope recognised by the antibody.

Immunofluorescence studies were carried out using the same antibody. In frozen sections of mouse cornea, the stroma stained positively for keratan sulphate, whereas costal cartilage sections were negative (results not shown).

General discussion

We have found that costal cartilage and intervertebral disc proteoglycans in the mouse and rat do not contain keratan sulphate. The major glycosaminoglycan of these tissues is chondroitin sulphate, with very small amounts of heparan sulphate. Our results confirm and extend studies on epiphyseal cartilage proteoglycans of achondroplasic (Kleinmann *et al.*, 1977) and brachyomorphic (Orkin *et al.*, 1976) mice, and on costal cartilage proteoglycans of Sprague–Dawley rats (Oohira & Nogami, 1980) which were essentially all degraded by chondroitinase ABC. The lack of mouse skeletal keratan sulphate probably contributes to the readiness with which antibodies to this glycosaminoglycan have been raised in mice after immunization with human articular cartilage proteoglycans (Caterson *et al.*, 1983) or pig articular chondrocytes (Zanetti *et al.*, 1985). Lack of skeletal KS II is not a general feature of rodent skeletal proteoglycans, as it has been demonstrated in guinea-pig costal cartilage and intervertebral disc (Lohmander *et al.*, 1973) and rabbit costal cartilage (R. M. Mason & R. Torabian, unpublished work; Masden *et al.*, 1983).

As both rat and mouse synthesize KS I, reasons for the absence of KS II synthesis may be sought by examining the difference in synthesis of keratan sulphate-containing proteoglycans in the two tissues. These reside in the type of core protein, and the glycosaminoglycan chain length, its degree of sulphation and its linkage to the core protein (Hirano *et al.*, 1961; Bhavanandan & Meyer, 1966; Seno *et al.*, 1965; Mathews & Cifonelli, 1965). KS I chains are attached to asparagine via an *N*-glycosylamine bond to *N*-acetylglucosamine (Nilsson *et al.*, 1983). KS II chains are attached *O*-glycosidically through *N*-acetylgalactosamine to serine or threonine. It seems likely that both KS I and KS II chains are built up on oligosaccharide precursors of either the *N*-glucosyl or *O*-mucin type respectively (De Luca *et al.*, 1980; Lohmander *et al.*, 1980), probably after trimming of the terminal mannose residues from the former.

Lack of KS II synthesis in skeletal tissues of mice and rats could arise for several different reasons. Genes for the UDP-sugar transferases may not be switched on in chondrocytes, or they may utilize different transferases, which are absent. Alternatively cartilage proteoglycans may contain structurally different linkage region oligosaccharides which do not permit elaboration of keratan sulphate chains.

It has been demonstrated that cartilage contains several different proteoglycan populations differing in chondroitin and keratan sulphate content and having chemically and immunologically distinct core proteins (Heinegård *et al.*, 1981, 1985). Therefore another possibility for lack of KS II synthesis may be a failure of mouse and rat chondrocytes to express the requisite core protein.

The amount of keratan sulphate in many cartilages increases with increasing age (Kaplan & Meyer, 1959; Mason & Wusteman, 1970; Simunek & Muir, 1972; Bayliss & Venn, 1980) and is accompanied by a decrease in the proportion of unsubstituted *O*-linked oligosaccharides on proteoglycans (Garg & Swann, 1981; Sweet *et al.*, 1979). These changes are most marked during tissue maturation (Inerot & Heinegård, 1983; Thonar & Sweet, 1981), and may be associated with the changing load-bearing nature of articular cartilages (Sweet *et al.*, 1977; Hascall, 1983) although this has been disputed (Roughley *et al.*, 1981).

It has been suggested that keratan sulphate

chains potentiate cartilage stiffness more than equivalent amounts of chondroitin sulphate (Kempson *et al.*, 1970). However, in pig, human and dog, the keratan sulphate content of the intervertebral disc proteoglycans is higher than in their cartilage proteoglycans (Stevens *et al.*, 1979; Ghosh *et al.*, 1977), yet the disc is thought to have lower compressive stiffness to enable it to absorb and redistribute the compressive forces applied to the spine whilst still allowing flexibility.

The absence of KS II from both cartilage and disc in mice and rats suggests that the size or concentration of the proteoglycan molecules may be more important than the keratan sulphate content in determining viscoelastic properties. This is in agreement with studies which show that the osmotic pressures of proteoglycans with varying chondroitin sulphate and keratan sulphate contents are similar (Comper & Laurent, 1978).

We thank Katharine Dyne for excellent technical assistance, Fiona Watt and Tony Ratcliffe for immunohistochemistry and radioimmunoassays, and the Arthritis and Rheumatism Council for financial support.

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