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# CHARACTERIZATION OF A PROTEOGLYCAN OF HIGH ELECTROPHORETIC MOBILITY

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## Summary

Proteoglycans from articular cartilage of young baboons (*Papio papio*) were fractionated on an associative density gradient. Material from the top of the gradient was shown to contain a proteoglycan of high electrophoretic mobility on large porosity gels. Associated and/or contaminating proteins were removed by ion-exchange chromatography on DEAE-cellulose and subsequent gel filtration on Sepharose 4B in the presence of 0.1% SDS. The electrophoretically homogeneous proteoglycan ($K_d$ 0.43 on Sepharose 4B SDS) contained 39.7% protein, was rich in aspartate, glutamate, leucine and glycine and had a GalN : GluN molar ratio of 3.87.

## Introduction

Proteoglycans, which are major components of hyaline cartilage, consist of a central protein core substituted with carbohydraye side chains [1—3].

Numerous studies have demonstrated the existence of marked polydispersity in samples of these substances [1,4—6]. Generally this polydispersity is due to variations in the length of the protein core and/or glycosaminoglycan side chains giving rise to a spectrum of continually changing substances. During electrophoresis, however, proteoglycans appear to exist in several main groups [7—9]. In proteoglycans from articular cartilage of young baboons three main bands may be identified [8,10]. In this paper, we describe the isolation and characterisation of the most rapidly migrating of the three bands.

Abbreviation: SDS, sodium dodecyl sulphate.

## Experimental procedures

*Materials*

All chemicals were of the best available grade: guanidine hydrochloride was from Schwarz/Mann; benzamidine HCl, 6-aminohexonoic acid, disodium EDTA and CsCl were from Merck; Sepharose 4B and Sepharose CL-4B were from Pharmacia; BioGel P2 and P30 were from Bio-Rad; DEAE-cellulose (DE-52) (microgranular form) was from Whatman; acrylamide and bisacrylamide were from Canalco; agarose (Indubiose A 37) was from Industrie Biologique Française.

*Analytical methods*

Uronic acid was determined by the carbazole-borosulfuric acid method using glucuronolactone as standard [11]. Sialic acid, as a monitor for keratan sulfate and the oligosaccharide [12], was determined by the Method of Jourdian et al. [13]. Amino acids and hexosamines were determined on a Beckman 116 amino acid analyzer after hydrolysis under nitrogen of samples in 6 M HCl for 24 h at 100°C, or in 4 M HCl for 4 h at 100°C for amino acids and hexosamines, respectively.

*Tissue*

Articular cartilages of young baboons (*Papio papio*) were removed immediately after death, frozen in solid $CO_2$ and stored at −40°C. After thawing to 4°C, the cartilage was separated from non-cartilagenous structures, diced finely with a scalpel and washed briefly in ice-cold 0.9% NaCl.

*Preparation of proteoglycans*

Cartilage pieces were transferred to and extracted in, 10-times their weight of 0.15 M NaCl [14], containing 0.01 M EDTA/0.1 M 6-aminohexanoid acid and 5 mM benzamidine HCl (pH 6.8) [15] by gentle shaking on a Fisher Roto-Rack for 24 h at 4°C. Extracts were filtered through glass wool, dialysed against 8 M urea in 0.05 M Tris-HCl (pH 6.8) and purified by chromatography on DEAE-cellulose as described below.

Other samples of tissue were transferred to and extracted in, 10-times their weight of 4 M guanidine hydrochloride in 0.05 M sodium acetate (pH 5.8) [16], containing the proteolytic enzyme inhibitors listed above, for 48 h at 4°C. The extract was filtered through glass wool and the clear filtrate dialysed against 9 vol. 0.05 M Tris-HCl (pH 5.8), containing inhibitors, at 4°C overnight. Solid CsCl was added to give a final density of 1.65 g/ml. Samples were subject to equilibrium density gradient centrifugation ($\rho_{av}$ = 1.65 g/ml, 130 000 × $g$ 48 h, 18°C, Beckman Type 60Ti rotor, 0.8—1 mg/ml uronic acid starting concentration). At the end of the run, centrifuge tubes were frozen in liquid nitrogen and cut into three fractions: $\rho_{av}$ 1.82 g/ml (fraction 1); 1.63 g/ml (fraction 2); 1.49 g/ml (fraction 3) (fractions $A_1$, $A_2$ and $A_3$ of Ref. 4). Each fraction was dialysed exhaustively against 8 M urea in 0.05 M Tris-HCl (pH 6.8), containing the enzyme inhibitors listed above.

*Purification of proteoglycans by ion-exchange chromatography on DEAE-cellulose [17]*

Samples prepared from the 0.15 M NaCl extract and from the density gradient fractions described above, were chromatographed on DEAE-cellulose ($Cl^-$ form) previously equilibrated with 8 M urea in 0.05 M Tris-HCl (pH 6.8) (about 1 mg uronate/g DEAE-cellulose; column dimensions 1.4 × 10 cm). Columns were eluted with three bed volumes of 8 M urea, 0.2 M NaCl in 8 M urea, 0.3 M NaCl in 8 M NaCl in 8 M urea and 2 M NaCl in 8 M urea, all in 0.05 M Tris-HCl (pH 6.8), containing enzyme inhibitors.

*Gel chromatography*

Analytical columns of Sepharose 4B and BioGel P30 (0.8 × 150 cm) were eluted with 0.5 M sodium acetate (pH 6.8) at room temperature (flow rate, 1 ml/h; 0.7-ml fractions). An analytical column of Sepharose CL-4B (0.8 × 150 cm) was eluted with 0.05 M sodium phosphate buffer in 0.1% sodium dodecyl sulfate (SDS) (pH 7.3) at 23°C [18] (flow rate, 0.6 ml/h; 0.7-ml fractions).

A sample of fraction 3 proteoglycan purified on DEAE-cellulose was dissolved in 4 M guanidine hydrochloride/0.05 M sodium acetate (pH 5.8) and allowed to stand at room temperature for 3 h. The sample was applied to a column of Sepharose CL-4B (0.8 × 150 cm) and eluted with the same buffer at room temperature. Fractions were collected as before and analysed for uronic acid.

All columns were calibrated with fraction 1 proteoglycan from baboon cartilage and glucuronolactone. Column effluents were analysed for uronic acid or sialic acid.

Alkaline-borohydride-treated samples were desalted on columns of BioGel P2 (1.5 × 25 cm) eluted with 0.5 M pyridine acetate (pH 7.0) [12].

*Electrophoretic techniques*

Samples were dialysed against 8 M urea in 0.05 M Tris-HCl (pH 6.8) before gel electrophoresis on large-pore composite polyacrylamide agarose gels. A modification [7] of the method of McDevitt and Muir [19] was used: gels consisted of 1.2% w/v acrylamide and 0.7% w/v agarose. Samples equivalent 1.2–1.5 μg hexuronic acid were layered on each gel and subject to electrophoresis at 5 mA/tube and 21 V/cm, at 4°C for 55 min. Duplicate gels were stained with 0.2% toluidine blue in 0.1 M acetic acid and with Coomassie brilliant blue. A sample of chondroitin sulfate as standard exhibited a constant mobility, greater than that of all proteoglycans.

Samples were also subject to SDS-polyacrylamide gel electrophoresis. Samples were reacted with 1% SDS/5% mercaptoethanol at 60°C for 1 h. 20–30-μl samples (50 μg dry weight) were applied to 7% polyacrylamide gels in 0.2 M phosphate buffer (pH 7), containing 0.2% SDS (0.3 cm × 7 cm). As a standard, 50 μg (dry weight) of material eluted with 0.2 M NaCl/8 M urea from DEAE-cellulose after application of total 4 M guanidine hydrochloride extract before centrifugation, was used with each experiment. Running buffer was 0.2 M phosphate, 0.2% SDS diluted 1 : 20 (pH 7). Electrophoresis was carried out at constant current of 5 mA/tube for 3 h 20 min at room temperature. Gels were stained with Coomassie brilliant blue and destained in 7% acetic acid before

being scanned at 570 nm in a Gilford Spectrophotometer fitted with a linear transport mechanism.

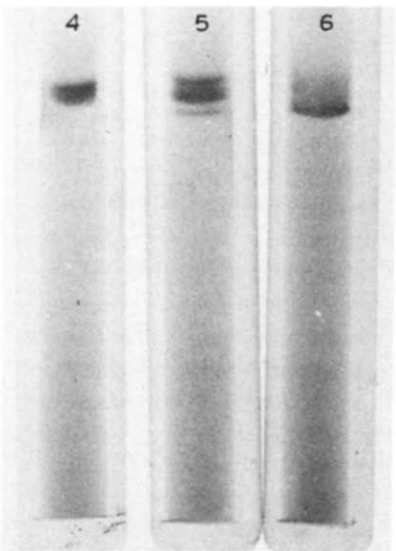
*Chemical and enzymatic degradation of proteoglycan*

A sample of fraction 3 proteoglycan was purified on DEAE-cellulose and treated with 0.05 M NaOH in 1 M sodium borohydride at 37°C for 36 h under nitrogen [3,20]. The borohydride was destroyed by the drop-wise addition of concentrated acetic acid and samples were then freeze-dried and desalted on BioGel P2.

Fraction 3 proteoglycan, purified on DEAE-cellulose, was digested with chondroitinase ABC (Sigma) (0.05 U/mg proteoglycan) in 0.1 M Tris-acetate (pH 7.3) at 37°C for 5 h. The material was subjected to ascending thin-layer chromatography on cellulose plates (Merck) using butyric acid/0.5 M $NH_4OH$ (5 : 3, v/v) as solvent. Plates were dried in a stream of air, stained in 1% toluidine blue in methanol, and then destained in methanol [21].

## Results

The proportion of the total uronate extracted with the associative solvent (0.15 M NaCl) was 7%, while 75% could be extracted with 4 M guanidine hydrochloride. Following equilibrium centrifugation, 68%, 13% and 19% (dry weight) of the loaded material was recovered from fractions 1, 2 and 3, respectively (as dry weight); uronic acid (% of total) was 91.2, 4.3 and 4.6, respec-



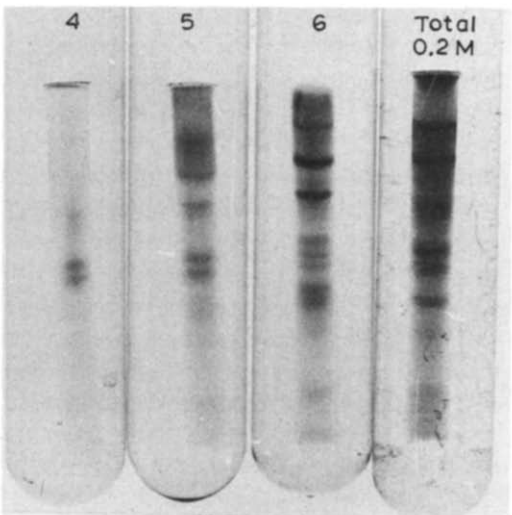




**Fig. 1. Electrophoretic separation on composite gels of proteoglycans from fractions 1, 2 and 3 purified on DEAE-cellulose. (Fractions 4, 5 and 6): 4, band I (predominant) and band II; 5, band I, band II (predominant) and band III; 6, predominantly band III.**

**Fig. 2. Electrophoretic analysis by SDS-polyacrylamide gel electrophoresis (7% polyacrylamide) of fractions 1, 2 and 3, purified on DEAE-cellulose (fractions 4, 5 and 6) and reduced with mercaptoethanol. 50 μg dry weight of each sample were used; as control 50 μg of the 0.2 M NaCl fraction of the DEAE-cellulose column.**

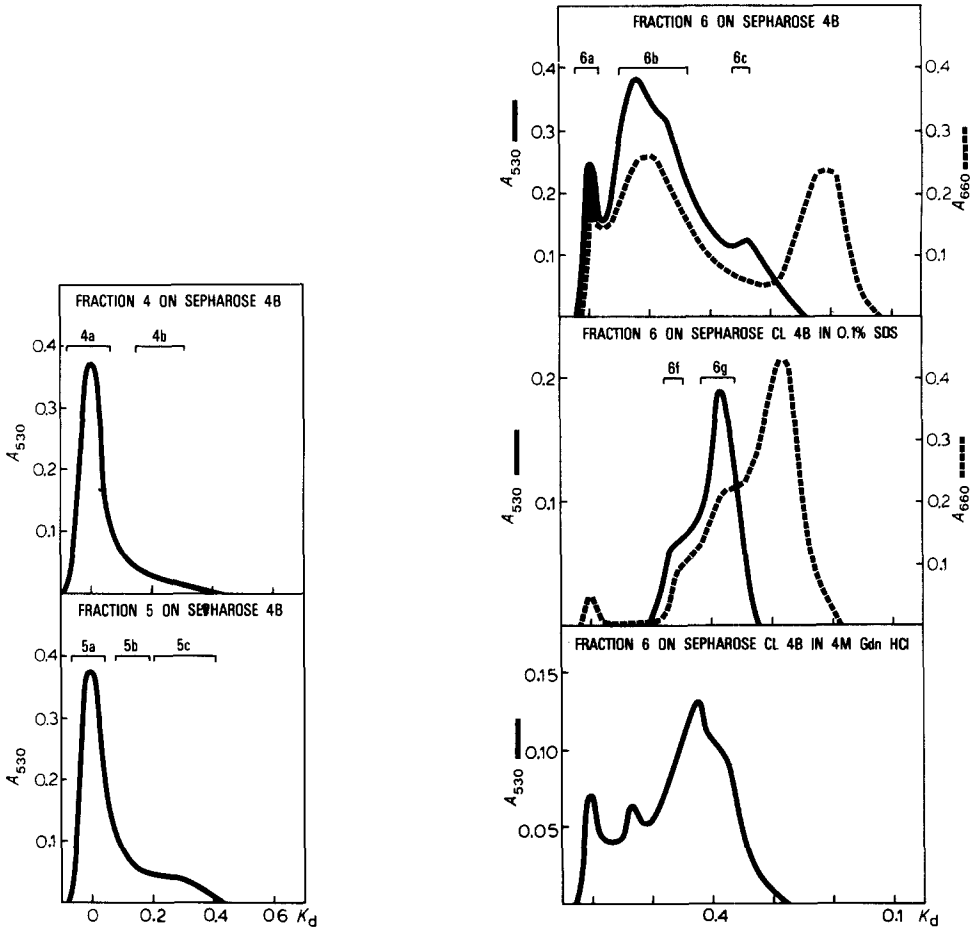
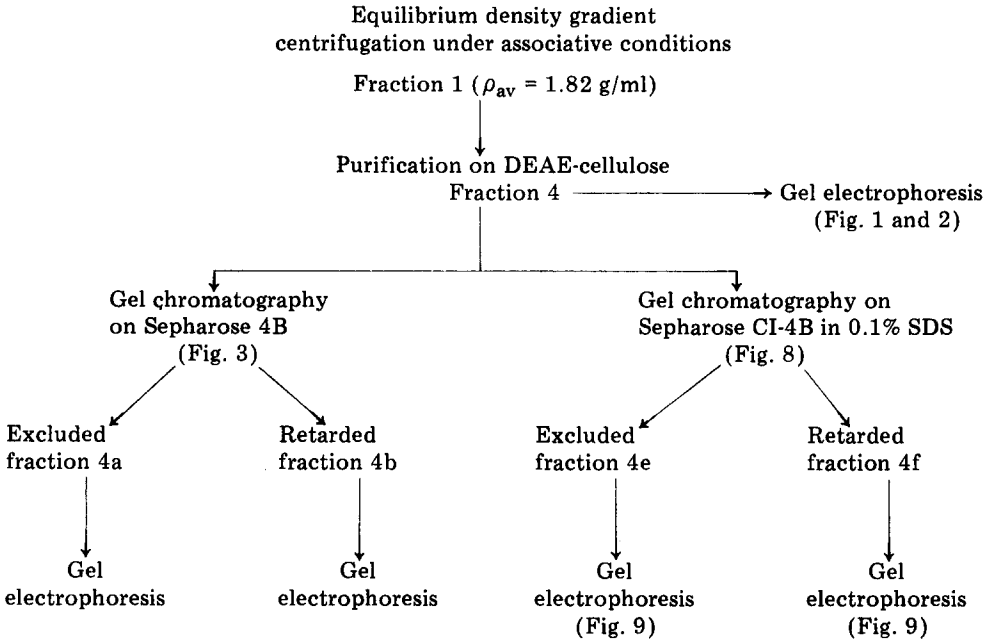



Fig. 3. Gel chromatography of fractions 4 and 5 on Sepharose 4B. Eluant, 0.5 M sodium acetate, pH 6.8.

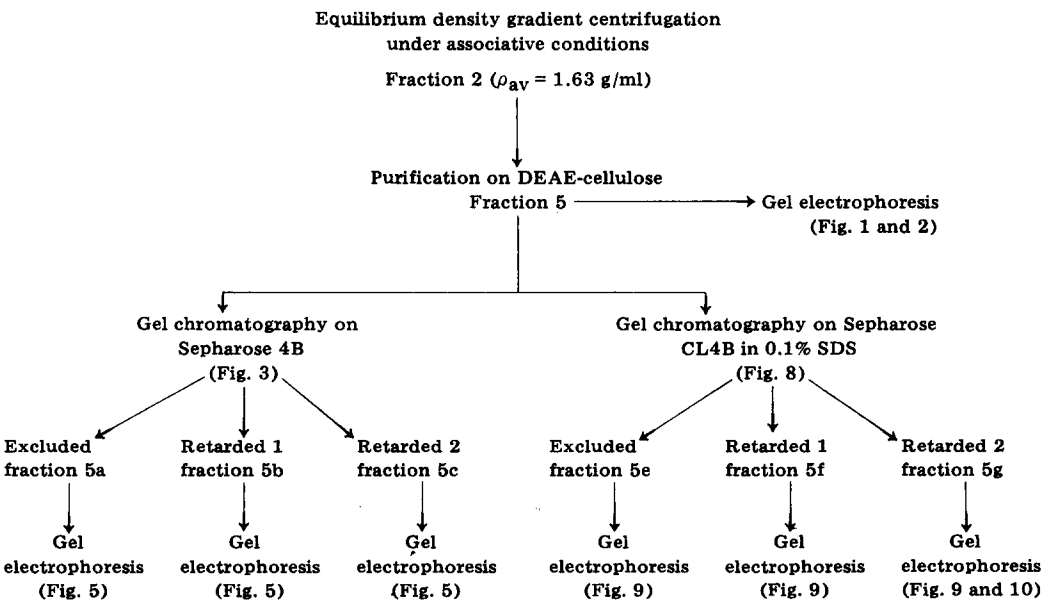
Fig. 4. Gel chromatography of fraction 6 on Sepharose 4B under varying conditions: (a) in 0.5 M sodium acetate, pH 6.8, 18°C, major included peak $K_d$ 0.2; second included peak $K_d$ 0.5; (b) in 0.05 M sodium phosphate, 0.1% SDS, pH 7.3, 23°C. Note single major peak $K_d$ 0.43; (c) in 4 M guanidinium chloride. Fractions marked 6a, 6b, 6c, 6f and 6g were recovered for further analysis by electrophoresis.

tively. (For further clarification see schemes Ia—c.)

Fractions 1, 2 and 3 were further purified by DEAE-cellulose ion-exchange chromatography. The purified fractions (4, 5 and 6) were assessed on composite (polyacrylamide/agarose) gels and on 7% SDS-polyacrylamide gels. As in previous studies [7—10], three distinct bands were noted on the large-pore composite gels: two wide bands close together (I and II) and a faster more discrete band (III). Bands I and II were strongly metachromatic; band III was less metachromatic and by contrast, stained well with Coomassie brilliant blue. Bands I and II were noted in fraction 4; band I, II and III (predominantly II) in fraction 5; predominantly band III in fraction 6 (Fig. 1). The 0.15 M NaCl extracted contained bands I and II (diffusely stained) and a strong band III. Comparative SDS-polyacrylamide gel electrophoresis of each sample indicated, despite equilibrium centrifugation (in comparatively high dilution) and sub-

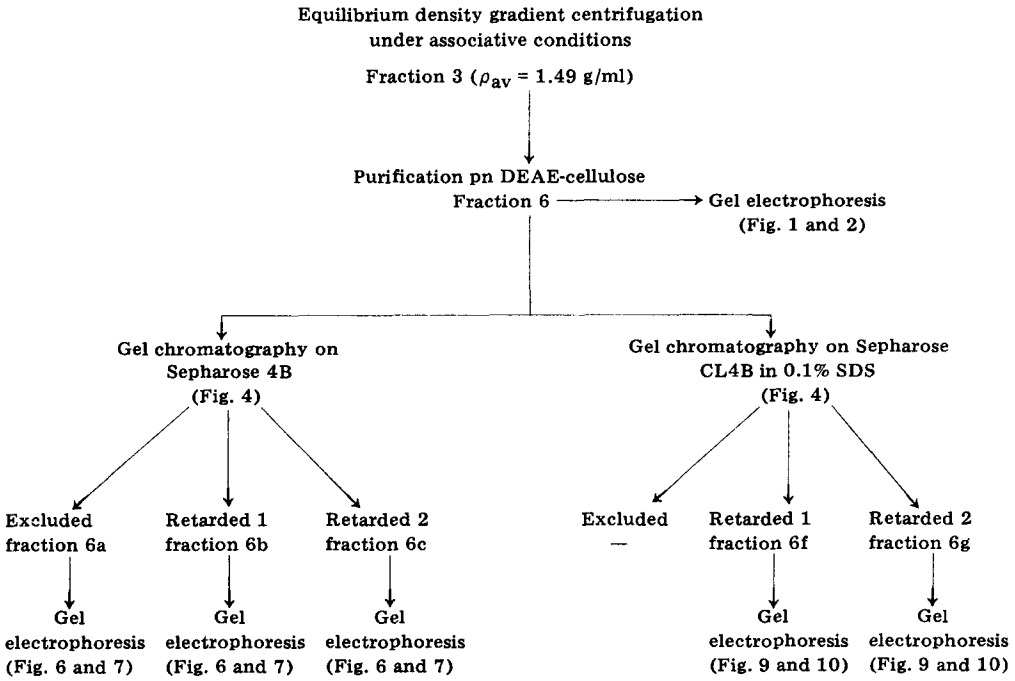



SCHEME Ia




SCHEME Ib

Scheme I. a. Representation of the separation of the proteoglycans (4 M guanidinium HCL extract of baboon articular cartilage). Fraction 1. b. Representation of the separation of the proteoglycans (4 M guanidinium HCl extract of baboon articular cartilage). Fraction 2. c. Representation of the separation of the proteoglycans (4 M guanidinium HCl extract of baboon articular cartilage). Fraction 3.




SCHEME Ic

sequent ion-exchange chromatography on DEAE-cellulose, the presence of significant protein contamination and/or association in fraction 5 and, particularly, in fraction 6. Small amounts of the two link proteins and of a slower migrating protein were noted in fraction 4 (Fig. 2).

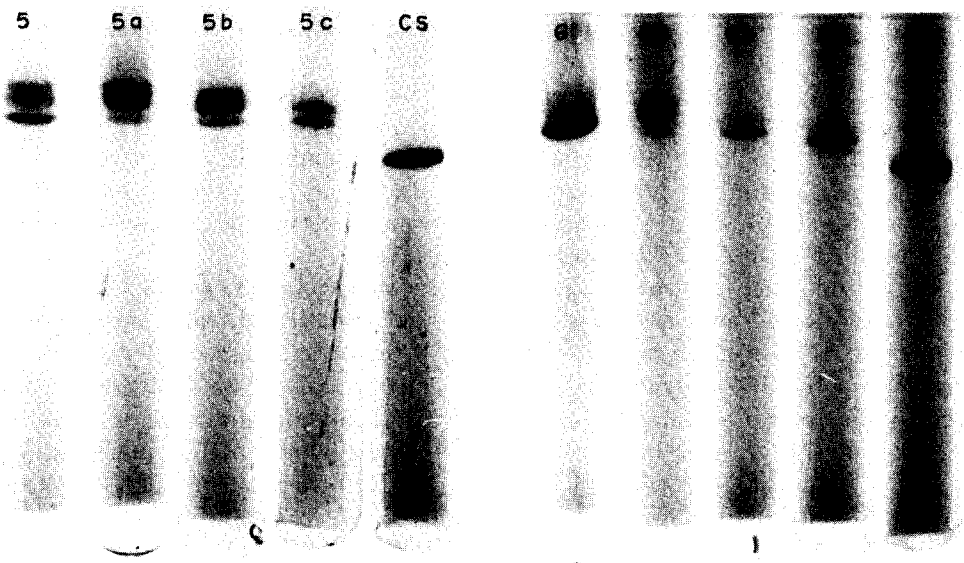
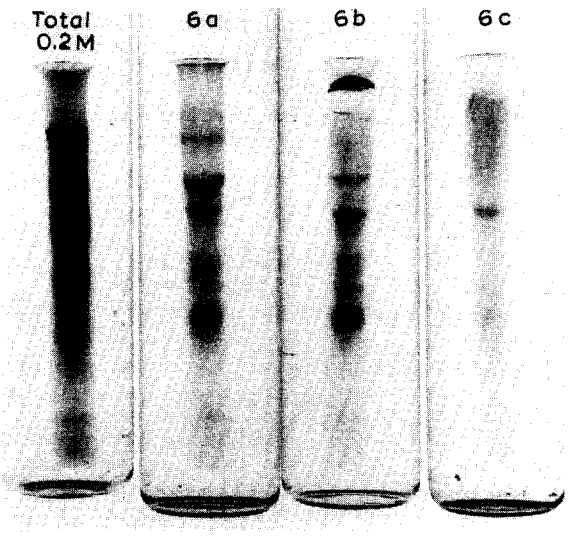



Fig. 5. Electrophoresis on composite gels of fraction 5 fractionated on Sepharose 4B (see Fig. 3).

Fig. 6. Electrophoresis on composite gels of fraction 6 fractionated on Sepharose 4B (see Fig. 4a).



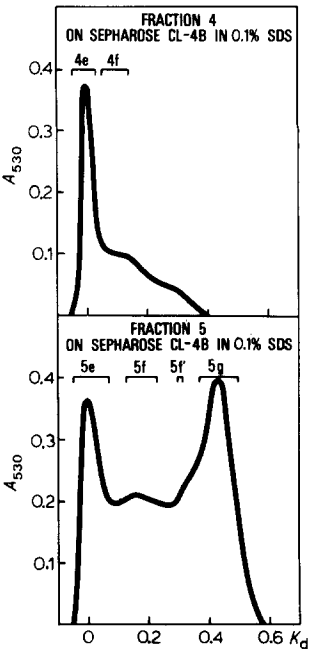




Fig. 7. SDS-polyacrylamide gel electrophoresis (7% polyacrylamide), fraction 6 fractionated on Sepharose 4B (see Fig. 4a), note presence of protein in fractions 6a and 6b and to a much lesser extent in fraction 6c.

Fig. 8. Gel filtration on Sepharose 4B in the presence of 0.1% SDS: (a) fraction 4; (b) fraction 5.

Fractions 4—6 were each subjected to chromatography on Sepharose 4B prior to further analysis by electrophoresis. Data in Fig. 3 indicate that the majority of the uronate-containing material of fractions 4 and 5 was excluded from the gel (fractions 4a and 5a). A small proportion of the latter fraction was included into the gel (fractions 4b, 5b and 5c). In the case of fraction 6, there was a small excluded peak (fraction 6a), with a definite included peak of $K_d$ 0.2 (fraction 6b), followed by a second smaller peak of $K_d$ 0.5 (fraction 6c) (Fig. 4). An amount of protein material eluted from the column near the void volume ($K_d$ 0.78). Each of the retarded uronate-positive peaks were recovered and rerun on Sepharose 4B with identical results.

Eluted material from each column was recovered as indicated by the bars in Figs. 3 and 4 and analysed by electrophoresis on composite gels. Material from Sepharose 4B chromatography of fraction 6 only was assessed for protein contamination by SDS-polyacrylamide gel electrophoresis. The excluded peak of fraction 5a contained bands I, II and III; fraction 5b contained bands II and III, predominantly II; fraction 5c contained bands II and III, equally stained (Fig. 5). Fraction 6a contained bands II and III, whereas fractions 6b and 6c contained only band III, although the band III, of the latter fraction was often slightly ahead of that of the former (Fig. 6). Definite protein contamination and/or association was noted in fractions 6a and 6b. In fraction 6c, there was only minor contamination by a single component, designated Peak 2 by Chaminade et al. [22] (Fig. 7).

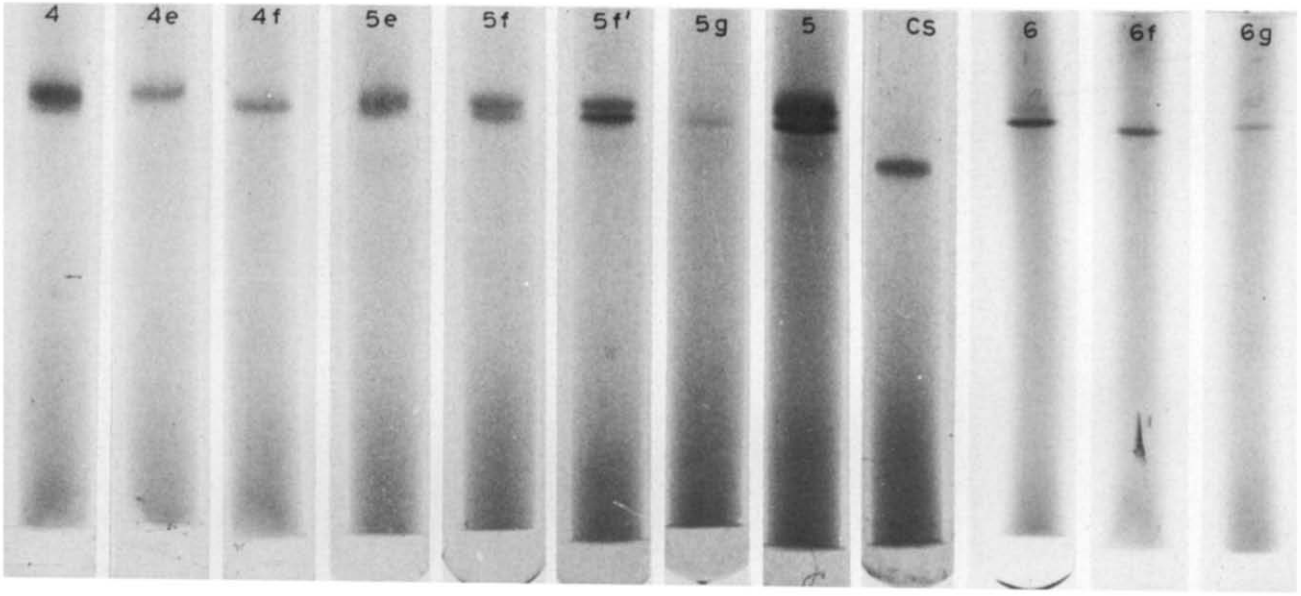
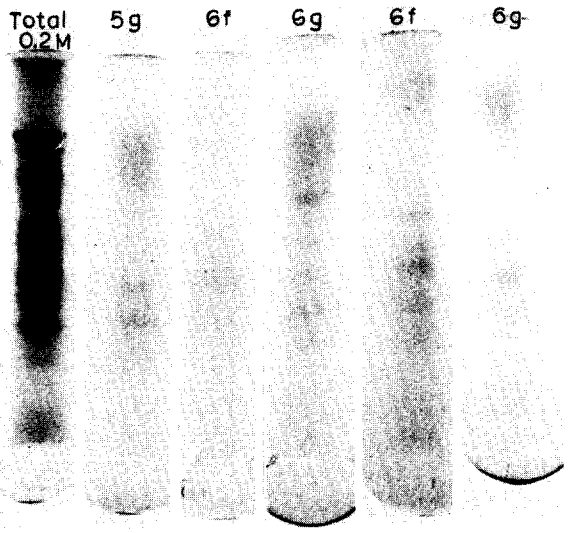



Fig. 9. Electrophoresis on composite gels of: (a) fraction 4; (b) fraction 5; (c) fraction 6 fractionated on Sepharose 4B in 0.05 M phosphate, 0.1% SDS (see Fig. 8 and 4b).



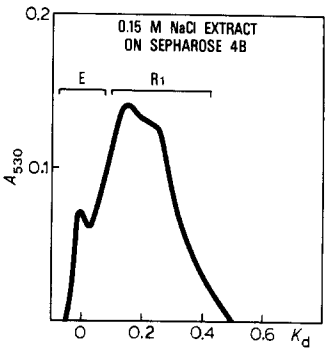




Fig. 10. 7% SDS-polyacrylamide gel electrophoresis of: fraction 5 fractionated on Sepharose 4B 0.1% SDS, fraction 5f (see Fig. 9b); fraction 6 fractionated on Sepharose 4B 0.1% SDS, fractions 6f and 6g. Total 0.2 M: 0.2 M NaCl fraction of the DEAE cellulose column.

Fig. 11. 0.15 M NaCl extract of total cartilage fractionated on Sepharose 4B after purification on DEAE cellulose.

In an attempt to remove associated or contaminating proteins from samples, fractions 4—6 were each treated with 1% SDS/5% mercaptoethanol before further analysis on a Sepharose CI-4B SDS column. The elution profiles of fraction 4 before and after treatment with SDS/mercaptoethanol were similar (Fig. 8). In the case of fraction 5, however, the proportion of excluded material was significantly reduced and replaced by 2 included peaks ($K_d$ 0.14 and 0.43, Fig. 8; fractions 5f and 5g, respectively). In the case of fraction 6, no material was excluded from the column after treatment with SDS/mercaptoethanol, but emerged as a single main peak of $K_d$ 0.43 as before (fraction 6g) (Fig. 4). Electrophoresis of the various excluded and retarded fractions of fraction 4 on composite gels was virtually as before (Fig. 9; band III alone was identified in fraction 5g (Fig. 9)). Analysis of fractions 6f and 6g on composite gels revealed the presence of band III only (Fig. 10); electrophoresis of these two fractions on SDS-polyacrylamide gels revealed only minimal protein association or contamination (Fig. 10).

The 0.15 M NaCl extract was chromatographed on Sepharose 4B after purification on DEAE-cellulose (Fig. 11). The material emerged as an excluded peak followed by a slightly retarded peak ($K_d$ 0.18) from which electrophoretically homogeneous band III was recovered (data not shown).

Samples of fractions 6b, 6c and 6g were recovered for compositional analysis (Table I). The amino acid composition of fraction 6g (which had been shown to be virtually free from contamination with other proteins) was different from that of 'standard' proteoglycans [1,2,9] and from that of the keratan sulphate-rich region [23,24]. It was comparatively rich in aspartic acid, leucine and valine

TABLE I

CHEMICAL COMPOSITION OF FRACTION 6 ISOLATED AFTER GEL FILTRATION ON SEPHAROSE 4B

n.d. represents not detected; amino acids are expressed in residues per 1000.

| | Fraction 6b | Fraction 6c | Fraction 6g |
|---|---|---|---|
| Amino acids | | | |
| Aspartic acid | 142 | 129 | 144 |
| Threonine | 51 | 40 | 35 |
| Serine | 59 | 51 | 66 |
| Glutamic acid | 123 | 120 | 140 |
| Proline | 86 | 112 | 67 |
| Glycine | 89 | 157 | 101 |
| Alanine | 59 | 75 | 81 |
| Half cystine | 29 | n.d. | 13 |
| Valine | 72 | 72 | 76 |
| Methionine | 5 | n.d. | 5 |
| Isoleucine | 33 | 37 | 37 |
| Leucine | 88 | 90 | 134 |
| Tyrosine | 19 | 15 | 15 |
| Phenylalanine | 31 | 31 | 31 |
| Lysine | 46 | 26 | 17 |
| Histidine | 22 | 12 | 13 |
| Arginine | 44 | 33 | 26 |
| Protein μmol/mg | 3.492 | 0.841 | 3.308 |
| Protein % w/w | 41.9% | 20.2% | 39.7% |
| Carbohydrate μg/mg | | | |
| Galactosamine | 80.4 | 101.5 | 47.7 |
| Glucosamine | 46.2 | 52.0 | 12.3 |
| Uronic acid | 76.0 | 70.5 | 45.0 |
| Sialic acid | 9.5 | n.d. | 4.8 |
| Molar ratio GalN/GluN | 1.74 | 2.01 | 3.87 |

and comparatively poor in serine and tyrosine. Summation of the amino acids showed it to be protein-rich (39.7%, w/w). The galactosamine : glucosamine molar ratio was only 3.87 (w/w). By contrast, fraction 6c contained only 20% (w/w) protein and was rich in aspartic acid, glycine and valine. Cysteic acid was not detectable and it is possible that the fraction represented a fragment of a larger molecule, or even a different species of proteoglycan. A sample of fraction 6b was treated with alkaline borohydride and the resulting product chromatographed on BioGel P30 [3,24]. Two sialic-acid-positive peaks ($K_d$ 0.5 and 0.7) were identified (data not shown) corresponding to keratan sulfate of 6 disaccharide units (on average) and the oligosaccharide, respectively [3]. Qualitative thin-layer chromatography on the unsaturated disaccharides, resulting from chondroitinase ABC lyase digestion of the same sample, showed that most of the chondroitin sulfate was 4-sulfated.

## Discussion

Previous work from these laboratories has indicated the existence of three electrophoretically distinct fractions of proteoglycan in articular cartilages [7—9]. The view that proteoglycans consist of a spectrum of continually chang-

ing substances rather than distinct sub-groups [23,25,26] may require modification in the light of the results presented in this and other studies [8,27]. The concept of polydispersity is applicable to at least two, but possibly three sub-groups, the largest and most populous of which (fraction A1 proteoglycan of Ref. 4) contains molecules which are recovered from the bottom of an associative gradient, are mostly capable of aggregation with hyaluronate and which may generally be said to consist of the three 'classical' regions of the core protein. By contrast the small proteoglycan of relatively low buoyant density which is especially characterized by its rapid electrophoretic mobility, is recovered from the upper part of an associative gradient, is protein-rich, is retarded by Sepharose 4B and is exceptional from a compositional point of view; it is predominantly 4-sulfated and appears to be associated with a number of other proteins, possibly via reducible bonds. As stated above, this component does exhibit a degree of polydispersity in that the reduced, SDS-treated form eluted from Sepharose 4B as a comparatively wide peak.

The possible ability of the component to be associated with other proteins is of interest. It might be argued that the associated proteins were no more than contaminants. Against this view must be belanced the facts that ion-exchange chromatography in the presence of 8 M urea did not ensure complete protein removal, that reduction with mercaptoethanol and treatment with SDS brought about a significant change in $K_d$ (Fig. 4) and that link proteins (Fig. 7) were found together with this proteoglycan in the absence of any other proteoglycan (i.e. bands I and II). Furthermore, repeated chromatography of numerous different preparations of fraction 6 gave identical results with the major peak eluting at $K_d$ 0.2. In view of the extreme condions required to dislodge the 'associated' proteins, it is possible that the proteoglycan is naturally thus associated in some way and that it may represent some form of carrier, especially for the link proteins.

There are certain points of similarity between the small proteoglycan described here and that described by Swann et al. [27], for example the high protein content and the high aspartic acid content. However there are compositional differences (valine, lysine and hexosamine contents) and it should be emphasized that in this study, rigorous electrophoretic control was employed at all stages. Furthermore the chances of contamination by small aggregating proteoglycans were reduced by the use of an initial associative gradient.

Low buoyant density proteoglycans have been isolated from embryonic chick cartilage [28], one of which is reducible with mercaptoethanol and which, it was suggested, could form aggregates in the presence of 4 M guanidine hydrochloride. In this regard a proportion of fraction 6 material was excluded from Sepharose 4B even in the presence of 4 M guanidine hydrochloride (Fig. 4).

The small proteoglycan described in this study is not identifiable in all forms of cartilage, at least by electrophoretic means. For example, it is not detectable in proteoglycans extracted from mature adult articular cartilage [29] and in unfractionated proteoglycans extracted from foetal baboon cartilage [7]. It has, however, been demonstrated in a wide variety of other cartilages [29] including elasmobranch cartilage (unpublished data), meniscus [10] and osteo-

arthrotic osteophytes [29]. This distribution will need to be taken into account in any hypothesis regarding its function.

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