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The Electrophoretic Heterogeneity of Bovine Nasal Cartilage Proteoglycans

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1. Proteoglycans were extracted from bovine nasal cartilage with 2.0M-$CaCl_2$ or with 0.15M-KCl followed by 2.0M-$CaCl_2$. Proteoglycan fractions were prepared from the extracts by density-gradient centrifugation in CsCl under 'associative' and 'dissociative' conditions. 2. The heterogeneity of the proteoglycan fractions was investigated by large-pore-gel electrophoresis. It was concluded that extracts made with 2.0M-$CaCl_2$ or sequential 2.0M-$CaCl_2$ contain two major species of proteoglycan 'subunit' of different hydrodynamic size, together with proteoglycan aggregates. Both 'subunits' have mobilities that are greater than those of proteoglycans obtained from pig articular cartilage [McDevitt & Muir (1971) *Anal. Biochem.* **44**, 612–622] and are therefore probably smaller in size than the latter. 3. Proteoglycan fractions isolated from cartilage extracted with 0.15M-KCl separated into two main components on large-pore-gel electrophoresis with mobilities greater than those of proteoglycans extracted with 2.0M-$CaCl_2$. Proteoglycans extracted at low ionic strength from bovine nasal cartilage are of similar hydrodynamic size to those extracted from pig articular cartilage under the same conditions [McDevitt & Muir (1971) *Anal. Biochem.* **44**, 612–622]. 4. The role of endogenous proteolytic enzymes in producing proteoglycan heterogeneity, particularly in low-ionic-strength cartilage extracts, is discussed. 5. Hyaluronic acid and 'link proteins' were present in the proteoglycan fraction separated from KCl extracts as well as in the fraction separated from $CaCl_2$ extracts. Hyaluronic acid can only be identified in proteoglycan fractions by large-pore-gel electrophoresis after proteolysis and further purification of the fraction. 6. Collagen was extracted by both salt solutions and was tentatively identified as type II. Small amounts of collagen appear to be associated with the proteoglycan-aggregate fraction from the high-ionic-strength extract but not with the corresponding fraction from the KCl extract.

The proteoglycans of hyaline cartilage are composed of a core protein to which side chains of chondroitin sulphate and keratan sulphate are attached to give a suggested brush-like structure (Mathews, 1971). Cartilage proteoglycans form large aggregates on interaction with hyaluronic acid and 'link proteins' (Hascall & Sajdera, 1969; Tsiganos *et al.*, 1972; Hascall & Heinegård, 1974*a*,*b*; Heinegård & Hascall, 1974; Hardingham & Muir, 1972, 1973*a*,*b*, 1974) which can be dissociated in concentrated solutions of guanidine hydrochloride (Hascall & Sajdera, 1969). Extraction of cartilage slices with high-ionic-strength salt solutions has proved effective in solubilizing proteoglycans from the tissue (Sajdera & Hascall, 1969; Mason & Mayes, 1973). On lowering the ionic strength of the extract by dialysis, proteoglycan aggregates re-form, and together with any non-aggregated proteoglycan they can be separated from proteins that are not specifically associated with them by centrifugation in a CsCl gradient (Hascall & Sajdera, 1969). This proteoglycan fraction will be referred to below as A1, following the nomenclature of Heinegård (1972*a*).

Centrifugation of A1 proteoglycan fractions in a CsCl density gradient in the presence of 4.0M-guanidine hydrochloride dissociates the aggregate and allows the separation of 'proteoglycan subunits' in the fraction of highest density (A1D1, $\rho > 1.53$ g/ml) from 'link protein' which is present in the fraction of lowest density (A1D3, $\rho < 1.45$ g/ml) (Hascall & Sajdera, 1969). Hyaluronic acid bands in the fraction of middle density (A1D2) (Hardingham & Muir, 1974). This method of fractionating high-ionic-strength salt extracts of cartilage has been used extensively to isolate proteoglycans and associated fractions for studies of their chemical, physical, interactive and immunological properties (Hascall & Sajdera, 1969, 1970; Rosenberg *et al.*, 1970*a*,*b*, 1973; Tsiganos *et al.*, 1971; Hascall & Riolo, 1972; Heinegård, 1972*a*,*b*; Di Ferrante *et al.*, 1972;

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Keiser *et al.*, 1972; Gregory, 1973; Mayes *et al.*, 1973; Pasternak *et al.*, 1974; Hardingham & Muir, 1974; Hascall & Heinegård, 1974*a*,*b*; Heinegård & Hascall, 1974).

Cartilage proteoglycans exhibit heterogeneity, which is attributed by some investigators to the occurrence of different classes of proteoglycan in the tissue (Tsiganos *et al.*, 1971) and by others to wide polydispersity of molecular size and structure of a single class (Hascall & Sajdera, 1970).

We report below the use of electrophoretic methods to detect heterogeneity in proteoglycans from bovine nasal cartilage. The results reveal both similarities to and differences from those obtained for pig articular-cartilage proteoglycans by McDevitt & Muir (1971). The protein components associated with each proteoglycan fraction were also examined by electrophoretic techniques.

A preliminary report of part of this work has been published (Roughley & Mason, 1975).

Experimental

Chemicals used were either AnalaR grade or the best grade commercially available. Analytical methods for the determination of hexuronic acid, glucosamine and galactosamine were those described previously (Mayes *et al.*, 1973). Hydroxyproline was determined by the method of Woessner (1961).

Preparation of proteoglycans (see Scheme 1)

Fresh bovine nasal cartilage was frozen at −20°C, cleaned of surrounding tissue and shredded with a Surform blade (Sajdera & Hascall, 1969). Cartilage slices were extracted with unbuffered 0.15M-KCl, pH6.5, at either 4°C or room temperature (20°C), as described previously (Mason & Mayes, 1973). After the extract had been filtered through a glass sinter (porosity no. 1), the filtrate was retained and the residue extracted in a similar manner with 2.0M-$CaCl_2$ buffered with sodium acetate (pH5.8, 0.5M in Na^+ ions). The extract was filtered as described above and the filtrate retained.

After dialysis against distilled water (1:19, v/v, 4°C), the filtrates were fractionated by centrifugation (48h) in CsCl (Sajdera & Hascall, 1969) under the conditions described previously (Mayes *et al.*, 1973). Three fractions were obtained, A1 ($\rho > 1.70$g/ml), which contained 90% or more of the total hexuronate content of the starting solution, A2 ($1.70 > \rho > 1.65$) and A3 ($\rho < 1.65$). After repeated dialysis against distilled water (4°C) the salt-free preparations were freeze-dried. A gel which formed on top of the CsCl density gradient during centrifugation was also collected.

Some cartilage samples were extracted directly with 2.0M-$CaCl_2$ buffered with sodium acetate (pH5.8, 0.05M in Na^+ ions) (see Scheme 1).

A1 proteoglycans were fractionated by centrifuging (48h) in a second density gradient, but under dissociative conditions in the presence of 4.0M-guanidine hydrochloride (Hascall & Sajdera, 1969). Three fractions were collected, A1D1 ($\rho > 1.53$), A1D2 ($1.53 > \rho > 1.43$) and A1D3 ($\rho < 1.43$). After repeated dialysis they were freeze-dried.

Separation of hyaluronic acid from proteoglycan fractions

Fractions were digested with papain (EC 3.4.22.2) (Scott, 1961), dialysed against distilled water (4°C), centrifuged in a bench centrifuge and the supernatants fractionated on cellulose columns saturated with aq. 1% (w/v) cetylpyridinium chloride (Antonopoulos *et al.*, 1961) at 30°C. Hyaluronic acid was eluted from the column by a 0.3M-NaCl solution containing 0.05% cetylpyridinium chloride.

Zone electrophoresis in a sucrose density gradient

Electrophoresis was carried out in sodium citrate buffer (0.02M in citrate, pH3.0) as described previously (Mayes *et al.*, 1973), but at 14–15°C. The conductivity of the buffer was measured at the same temperature, to calculate the electrophoretic mobility, m, of a component. Calculations of m should take account of differences between the scanning speed of the electrophoresis column and the chart speed of the instrument used to record the scan. Our previous results should be multiplied by a scaling factor of 1.299 to adjust for this.

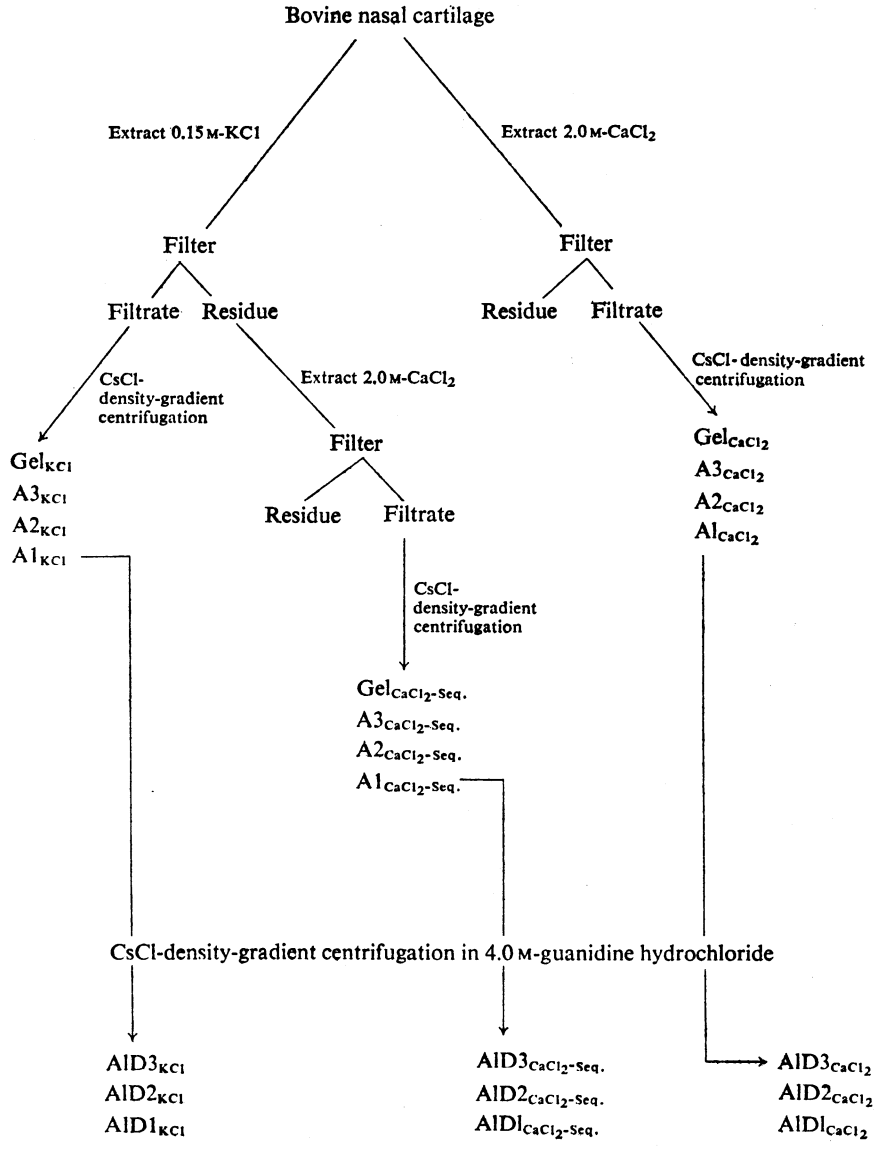
Large-pore-gel electrophoresis

Electrophoresis of proteoglycan samples [20μg in 25μl of 40% (w/v) sucrose containing Bromophenol Blue] on composite gels of polyacrylamide (1.2%, w/v) and agarose (0.6%, w/v) was performed by the method of McDevitt & Muir (1971) with the following modification. The upper portion was sliced off to leave a gel 7.0cm long with a flat surface, which was then supported on a 1cm 5% (w/v) polyacrylamide 'plug'. The operating voltage was 12V/cm with a current of 4mA/tube.

Gels stained with Toluidine Blue (McDevitt & Muir, 1971) were scanned at 520nm by using the linear transporter facility (0.5cm/min) of a Gilford 240 spectrophotometer with a slit-width of 0.05cm. The recorder chart was operated at 1.0cm/min.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in a discontinuous buffer system

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis with a sulphate/borate discontinuous buffer system, as described by Neville (1971), was used to investigate the nature of protein components



Scheme 1. *Extraction and fractionation of proteoglycans from bovine nasal cartilage*

Details of the extraction procedures and CsCl-density-gradient centrifugation are given in the Experimental section. Fractions derived from density gradients are designated 'A' where the centrifugation was carried out under associative conditions. Subfractions of 'A' fractions were derived from a second density-gradient centrifugation carried out under dissociative conditions and are designated 'AD'. The subscript, $CaCl_2$-Seq., distinguishes proteoglycan fractions derived from a sequential extraction of the tissue with $CaCl_2$ after extraction with KCl from fractions derived from single $CaCl_2$ extractions.

in the fractions. The pore size of the polyacrylamide gel was too small to allow proteoglycans to enter the gel.

Electrophoresis of samples and proteins of accurately known polypeptide-chain molecular weight was performed on both 7% (w/v) and 11% (w/v) polyacrylamide separating gels having a ratio of methylenebisacrylamide/acrylamide of 1:100. The compositions of the upper reservoir buffer (pH 8.64), the stacking gel (1 cm × 0.5 cm), separating gel

(7cm×0.5cm) and lower reservoir buffer (pH9.50) were as specified by Neville (1971).

Before electrophoresis, freeze-dried fractions were dissolved in water (2mg/ml) and a portion (20μl) was added to an equal volume of a denaturing solution [upper reservoir buffer, 25% (v/v); glycerol, 50% (v/v); water 24% (v/v); 2-mercaptoethanol 1% (v/v); sodium dodecyl sulphate, 2% (w/v); Bromophenol Blue, 0.01% (w/v)]. After heating (100°C, 2min), 20μl of the mixture was layered on the top of the stacking gel.

Insoluble gels (0.1g wet wt./ml), which formed at the top of the gradient during density-gradient centrifugation of cartilage extracts under associative conditions (see above), were dissolved in acetate buffer [pH5.8, 0.05M in Na^+ ions, containing 2% (w/v) sodium dodecyl sulphate] by heating (100°C, 2min), vigorous mixing and reheating (100°C, 2min). A portion (20μl) was then mixed with the denaturing solution (20μl) and treated as described above.

Electrophoresis was carried out at room temperature with a current of 2mA/tube for 70min, when the Bromophenol Blue band had migrated approx. 5cm. Gels were stained with Coomassie Brilliant Blue overnight and then destained as described by Weber *et al.* (1972). Mobilities of bands stained by the dye were calculated with reference to the Bromophenol Blue band, either by direct measurement of the gel or from densitometric scans. Gels were scanned as described above but at a wavelength of 580nm.

Acid-soluble collagen was prepared from rat skin by the method of Werb & Burleigh (1974), denatured and subjected to electrophoresis in the discontinuous buffer system to serve as a reference for collagen polypeptides.

Micro-zone electrophoresis on cellulose acetate membranes

Proteoglycan fractions were digested with papain and applied (0.1mg/10μl) to cellulose acetate membranes soaked in a calcium acetate buffer [pH7.25, 0.2M in acetate ions, diluted with ethylene glycol (3:2, v/v)] and subjected to electrophoresis, as described by Larsson *et al.* (1973). International standard glycosaminoglycan preparations were supplied by Dr. M. B. Mathews, University of Chicago, Chicago, IL, U.S.A. Membranes were stained with Toluidine Blue. These analyses were kindly carried out by Dr. K. E. Keuttner, Department of Orthopaedic Surgery, Rush-Presbyterian-St. Lukes Medical Centre, Chicago, IL 60612, U.S.A.

Results and Discussion

Yield of proteoglycan fractions

The low-ionic-strength extracts contained 15% and the high-ionic-strength extracts 83% of the total uronic acid in the tissue, in agreement with previous results (Mason & Mayes, 1973). The sequential procedure solubilized 15% of the total uronic acid in the KCl extract and a further 71% in the $CaCl_2$ extract.

After CsCl-density-gradient centrifugation of either low-ionic-strength- or high-ionic-strength-extracted material, 90% or more of the uronic acid present in the extract was recovered in the A1 fraction. Subsequent fractionation of A1 fractions by centrifugation under dissociative conditions yielded three subfractions, of which the most dense (A1D1) contained 90% of the total uronic acid in the tube. These results are in agreement with those published previously (e.g. Hascall & Sajdera, 1969; Mayes *et al.*, 1973; Hardingham & Muir, 1974). A1D1 fractions also accounted for approx. 90% (w/w) of A1 fractions obtained from either low- or high-ionic-strength extracts. The A1D2 and A1D3 fractions accounted for 6–10% (w/w) and 3–4% (w/w) respectively of A1 fractions from each type of extract.

Zone electrophoresis of proteoglycans in a sucrose density gradient

The electrophoretic properties of the A1 and A1D fractions from the KCl and sequential $CaCl_2$ cartilage extracts were similar to one another and to those reported previously for cartilage proteoglycans (Mayes *et al.*, 1973), each fraction having a major component with mobility (m) 0.58×10^{-10}–0.61×10^{-10} $m^2\cdot s^{-1}\cdot V^{-1}$. Under the conditions of the electrophoresis (pH3.0) all the proteoglycan species present would be expected to be in a dissociated state (Hascall & Sajdera, 1969).

In most preparations a minor component ($m = 0.33\times10^{-10}$ $m^2\cdot s^{-1}\cdot V^{-1}$) was just detectable, separating from the main component after 60min electrophoresis. The identity of this component is unknown.

The observations are in keeping with those of Mashburn & Hoffman (1971), who found that different proteoglycan fractions from bovine cartilages migrate to a characteristic peak area in free-flow electrophoresis experiments.

Large-pore-gel electrophoresis of proteoglycans

The results for all the proteoglycan fractions are shown for comparison in Fig. 1. Relative electrophoretic mobilities are given in Table 1. Densitometric scans of three fractions are shown in Fig. 2. Ideally, the latter method provides the most objective method for assessing the electrophoresis. However, the close proximity of components of different electrophoretic mobility on the gels results in 'shoulders' being recorded on densitometric scans, rather than clear-cut separations. At lower loading concentrations of proteoglycan, better separations

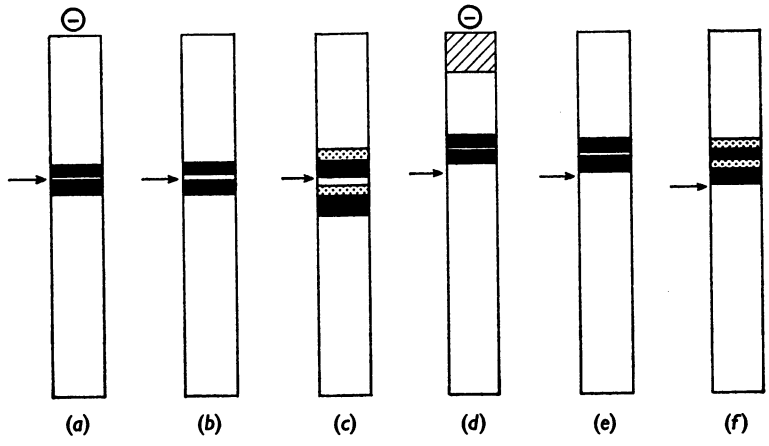


Fig. 1. *Large-pore-gel electrophoresis of proteoglycan fractions*

Proteoglycan fractions isolated from bovine nasal cartilage were subjected to electrophoresis through composite agarose/polyacrylamide gels, which were then stained with Toluidine Blue. The Figure shows a scale diagram of the position of bands stained by the dye. Densitometric scans of three of the gels are shown in Fig. 2. Bromophenol Blue was included in the sample as a reference compound and its position in the gel, after electrophoresis, is indicated by the arrow. Bromophenol Blue diffuses out of the gel during staining and destaining. (*a*) $A1_{KCl}$; (*b*) $A1D1_{KCl}$; (*c*) $A1D2_{KCl}$; (*d*) $A1_{CaCl_2\text{-}Seq.}$; (*e*) $A1D1_{CaCl_2\text{-}Seq.}$; (*f*) $A1D2_{CaCl_2\text{-}Seq.}$. For discussion of the results, see the text.

were recorded (e.g. Fig. 2*b*). Both the $A1_{KCl}$ and $A1D1_{KCl}$ fractions separate into two bands with corresponding mobilities. The $A1D2_{KCl}$ fraction also separates into two bands, but band (1) (see Table 1*a*) has a greater mobility than the corresponding band in the other fractions, suggesting that the middle fraction contains the smallest proteoglycans, as there is an inverse relationship between mobility and size of macromolecules (McDevitt & Muir, 1971). Both bands in the $A1D2_{KCl}$ fashion show some 'tailing' in the gel.

Chondroitin sulphate has a relative mobility of 1.3 under the same conditions and therefore the components giving rise to the bands are likely to have a multichain glycosaminoglycan structure.

Trace quantities of glycosaminoglycan could be detected in fraction $A1D3_{KCl}$ by the micro-zone electrophoresis method (Fig. 3), but not by the large-pore-gel electrophoresis method.

Fraction $A1_{CaCl_2\text{-}Seq.}$ separates into three components. One component (band 3) has a very low mobility and is not present in fraction $A1D1_{CaCl_2\text{-}Seq.}$, and can therefore be attributed to proteoglycan aggregate (see Table 1, Figs. 1 and 2). Bands 1 and 2 have corresponding mobilities in both fractions and are probably due to dissociated proteoglycans.

Fraction $A1D2_{CaCl_2\text{-}Seq.}$ also separates into two components, one (band 1) having a greater mobility than any component in the other fractions, the other (band 2) corresponding to band 2 of fraction $A1D1_{CaCl_2\text{-}Seq.}$. Thus the smallest proteoglycans in the high-ionic-strength extract are found in the less-dense middle fraction.

Glycosaminoglycan could be detected only in the $A1D3_{CaCl_2\text{-}Seq.}$ fraction by the micro-zone electrophoresis technique (Fig. 3).

No differences were observed in the mobilities of the proteoglycan fractions isolated from extracts made at 4°C compared with those made at room temperature.

The mobility of band (1) of fraction $A1_{KCl}$ is sufficiently different from that of bands (1) and (2) of fraction $A1_{CaCl_2\text{-}Seq.}$ to predict that electrophoresis of non-sequentially extracted $A1_{CaCl_2}$ fractions would show all three bands. However, $A1_{CaCl_2}$ fractions have the same appearance on the gels as $A1_{CaCl_2\text{-}Seq.}$ fractions.

This apparent discrepancy may arise from two sources. First, minor components in a fraction cannot be detected by the method. When various amounts of $A1D1_{KCl}$ and $A1D1_{CaCl_2\text{-}Seq.}$ fractions were mixed together before electrophoresis, band (1) of the former could not be detected when that fraction formed 20% (w/w) or less of the total proteoglycan, whereas with larger amounts [e.g. 40:60 (w/w) fraction $A1D1_{KCl}$/fraction $A1D1_{CaCl_2\text{-}Seq.}$] three bands were visible in the gel. Proteoglycans solubilized by 0.15M-KCl account for less than 20% of the total extractable proteoglycan and on this evidence would therefore not be detectable by large-pore-gel electrophoresis of fractions from straight $CaCl_2$ extracts.

Table 1. *Electrophoretic properties of proteoglycan fractions from bovine nasal cartilage*

(*a*) Relative electrophoretic mobilities (R_x) of fractions to Bromophenol Blue; (*b*) electrophoretic mobilities as a percentage of chondroitin sulphate mobility compared with porcine proteoglycan fractions (McDevitt & Muir, 1971). Proteoglycan fractions were subjected to electrophoresis through composite agarose/polyacrylamide gels and stained with Toluidine Blue (Figs. 1 and 2). For details of proteoglycan preparation and electrophoresis, see the text. Data for porcine fractions are from McDevitt & Muir (1971).

(*a*) Proteoglycan fraction	Band	Relative mobility (R_x)
KCl extract A1	(1)	1.05
	(2)	0.95
A1D1	(1)	1.05
	(2)	0.95
A1D2	(1)	1.19
	(2)	0.96
$CaCl_2$-Seq. extract A1	(1)	0.89
	(2)	0.80
	(3)	0.21
A1D1	(1)	0.91
	(2)	0.81
A1D2	(1)	0.95
	(2)	0.79

	Mobility relative to chondroitin sulphate	
(*b*) Preparation	Bovine fractions	Porcine fractions
Chondroitin sulphate	100*	100†
Trypsin-treated proteoglycan	89.2‡	89.7§
Low-ionic-strength-extract proteoglycan	73.1, 80.8‖	74.0¶
High-ionic-strength-extract proteoglycan	16.2, 61.5, 68.5**	0, 37.0, 41.7††

* Prepared by papain digestion of bovine $A1D1_{CaCl_2\text{-}Seq.}$ fraction (P. J. Roughley, unpublished work).

† Prepared by papain digestion of pig knee-joint cartilage.

‡ Prepared by trypsin digestion of bovine $A1D1_{CaCl_2\text{-}Seq.}$ fraction (P. J. Roughley, unpublished work).

§ Prepared by trypsin digestion of pig laryngeal cartilage.

‖ Bovine $A1_{KCl}$ fraction.

¶ Prepared by 9-aminoacridine precipitation of 0.15M-sodium acetate extract of pig knee cartilage.

** Bovine $A1_{CaCl_2\text{-}Seq.}$ fraction.

†† Prepared by 9-aminoacridine precipitation of 2.0M-$CaCl_2$ sequential extract of pig knee cartilage.

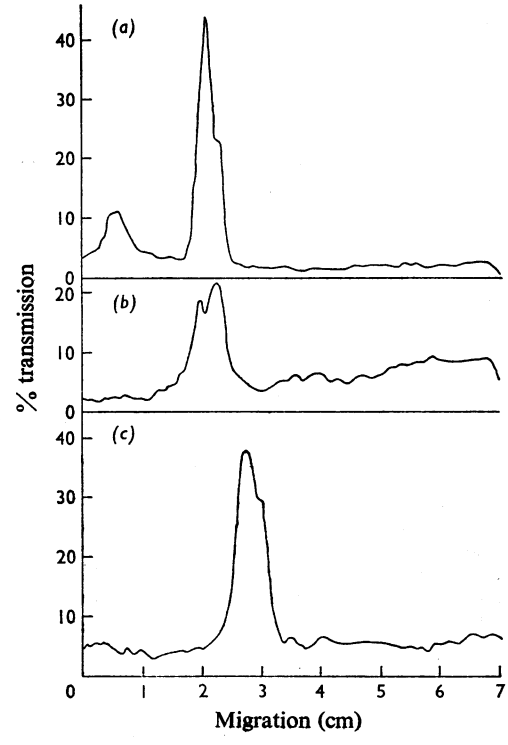


Fig. 2. *Densitometric scans of large-pore gels*

Densitometric scans of three proteoglycan fractions after electrophoresis on large-pore gels are shown. The gels were stained with Toluidine Blue and scanned in a Gilford 240 spectrophotometer at 520nm as described in the text. (*a*) $A1_{CaCl_2\text{-}Seq.}$; (*b*) $A1D1_{CaCl_2\text{-}Seq.}$; (*c*) $A1D1_{KCl}$. Results for the electrophoresis of the whole series of proteoglycan fractions referred to in the text are shown in Fig. 1 and Table 1.

Secondly, at least some of the small proteoglycans solubilized by KCl extraction may be produced by limited action of endogenous proteinases, capable of functioning in iso-osmotic salt solutions, but not in high-ionic-strength solutions (see the Discussion section). If this is so, straight $CaCl_2$ extracts would not contain these species.

Inability to detect minor components on large-pore gels also precludes the direct identification of hyaluronic acid in proteoglycan fractions. Hyaluronic acid constitutes 0.4–0.8% (w/w) of the bovine nasal A1 fraction (Hascall & Heinegård, 1974*a*) and bands predominantly in the A1D2 fraction on dissociation (Hardingham & Muir, 1974). It was detected in dissociated fractions from both extracts by the micro-zone method (Fig. 3), but only on large-pore gels after further purification of the fraction (see the Experimental section) and by performing the de-staining procedure in water. The bands had the same relative mobility (R_x 0.89) as that of hyaluronic acid (glucosamine/galactosamine/hexuronic acid, 1.0:0.0:0.9, by mol) isolated by the same method

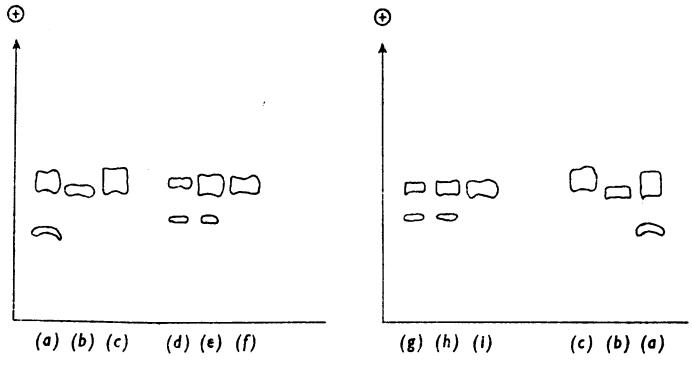


Fig. 3. *Micro-zone electrophoresis of papain-digested proteoglycan fractions*

Samples (100 μg) of papain-digested proteoglycan samples were subjected to electrophoresis on cellulose acetate membranes as described in the Experimental section. A diagrammatic representation of the separation of glycosaminoglycans, stained with Toluidine Blue, after electrophoresis, is shown. (*a*) Standard hyaluronic acid and chondroitin 6-sulphate; (*b*) chondroitin 4-sulphate; (*c*) keratan sulphate; (*d*) $A1D3_{KCl}$; (*e*) $A1D2_{KCl}$; (*f*) $A1D1_{KCl}$; (*g*) $A1D3_{CaCl_2-Seq.}$; (*h*) $A1D2_{CaCl_2-Seq.}$; (*i*) $A1D1_{CaCl_2-Seq.}$. Bands corresponding to chondroitin sulphate and keratan sulphate stained with the following relative intensities for the same quantities of proteoglycan applied; A1D1 (+++); A1D2 (++); A1D3 (+). Bands corresponding to hyaluronic acid were only faintly stained (±).

from 'fraction S' of a $LaCl_3$ cartilage extract (Mason & Roughley, 1974).

Identification of collagen in cartilage extracts

Collagen was detected in several fractions (see Scheme 1) by hydroxyproline analysis. The KCl/gel and $CaCl_2$-Seq./gel contained 0.9 and 1.3% (w/w) collagen of the wet gel respectively. Densitometric scans of both 7 and 11% sodium dodecyl sulphate/polyacrylamide gels of these fractions (Figs. 4 and 5) showed a major component with a mobility corresponding to an α_1-collagen polypeptide and a trace component with a β_{11}-polypeptide mobility. Both remained after digestion with trypsin, but α_2-type polypeptide components could not be detected (P. J. Roughley, unpublished work). The collagen present was therefore tentatively identified as type II.

α_1-Type polypeptides were also present in fraction $A3_{CaCl_2-Seq.}$ but not in fractions $A3_{KCl}$. The latter fraction contains a major polypeptide component which is also prominent in the corresponding gel fraction.

The major polypeptide components associated with the proteoglycan solubilized by KCl and $CaCl_2$ extracts were separated in the A1D3 fractions and identified as the link proteins, as reported previously (Roughley & Mason, 1975). However, fraction $A1D3_{CaCl_2-Seq.}$ contained 2.9% (w/w) collagen of the freeze-dried fraction as determined by hydroxyproline assay, and a minor polypeptide band with an α_1-component mobility was detected on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 5). Collagen could not be detected in $A1D3_{KCl}$ fraction (Fig. 4).

Discussion

Large-pore-gel electrophoresis is not capable of detecting minor components in heterogeneous proteoglycan fractions. Nevertheless, several different proteoglycan bands are separated in fractions obtained from sequential extracts of bovine nasal cartilage. This apparent heterogeneity could arise from two sources. Either naturally occurring proteoglycans in the tissue comprise a single polydisperse series (Hascall & Sajdera, 1970), which undergoes limited proteolysis during extraction resulting in apparent heterogeneity or, alternatively, several species with different core-protein structures may be present in the cartilage. The latter hypothesis is supported by investigations on pig cartilage proteoglycans by Muir and her colleagues, who found that fractions of different hydrodynamic size have different amino acid composition, *N*-terminal analysis, antigenic determinants, buoyant density and ability to form aggregates (see, for example, Tsiganos & Muir, 1969; Tsiganos *et al.*, 1971; Brandt & Muir, 1971*a*,*b*; Brandt *et al.*, 1973; Hardingham & Muir, 1974).

After degrading 'proteoglycan subunit' from bovine tracheal cartilage with hyaluronidase to remove chondroitin sulphate, Heinegård (1972*b*)

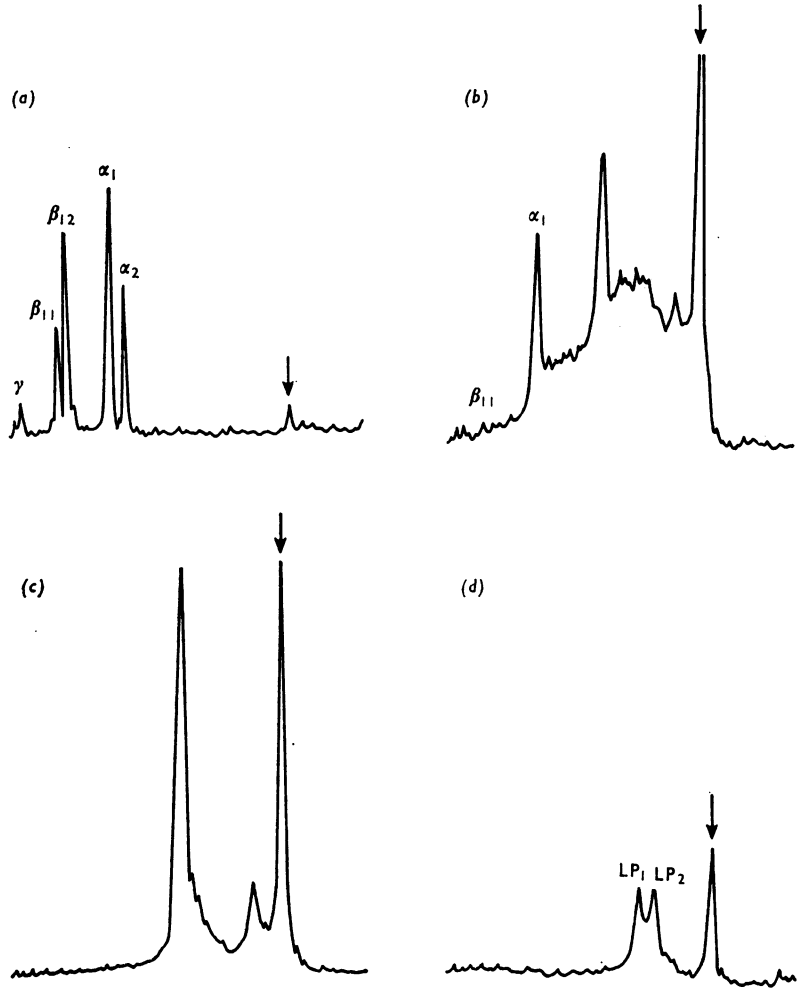


Fig. 4. *Sodium dodecyl sulphate/polyacrylamide-disc-gel electrophoresis of proteoglycan fractions obtained from* 0.15M-*KCl extracts of bovine nasal cartilage*

Proteoglycan fractions were isolated as described in the text. Densitometric scans of 7% (w/v) polyacrylamide gels after electrophoresis of various fractions are shown. (*a*) Acid-soluble rat skin collagen; (*b*) gel $_{KCl}$; (*c*) $A3_{KCl}$; (*d*) $A1D3_{KCl}$. α, β and γ refer to the positions in the gel to which the various collagen polypeptides migrate. LP_1 and LP_2 refer to the bands given by reduced 'link proteins'. The arrows mark the position of the Bromophenol Blue band.

isolated three fractions from the digest, each with different amino acid compositions. Similarly, Baxter & Muir (1975) isolated several core-protein preparations of different hydrodynamic size from pig laryngeal and bovine nasal cartilage.

Although all this evidence indicates heterogeneity of cartilage proteoglycans on the basis of the biosynthesis of two of more different core proteins, it must nevertheless be considered that at some stage during the experimental procedures, a limited proteolysis may have occurred producing artifacts. In recent experiments (J. P. Pearson & R. M. Mason, unpublished work) we have attempted to examine this possibility by extracting cartilage at 1°C, pH6.5, in the presence of phenylmethanesulphonyl fluoride, sodium iodoacetate, α-aminohexanoic acid, benzamidine hydrochloride, pancreatic trypsin inhibitor and EDTA. Sequential high-ionic-strength extracts were made with 4.0M-guanidine hydrochloride, which is even more likely

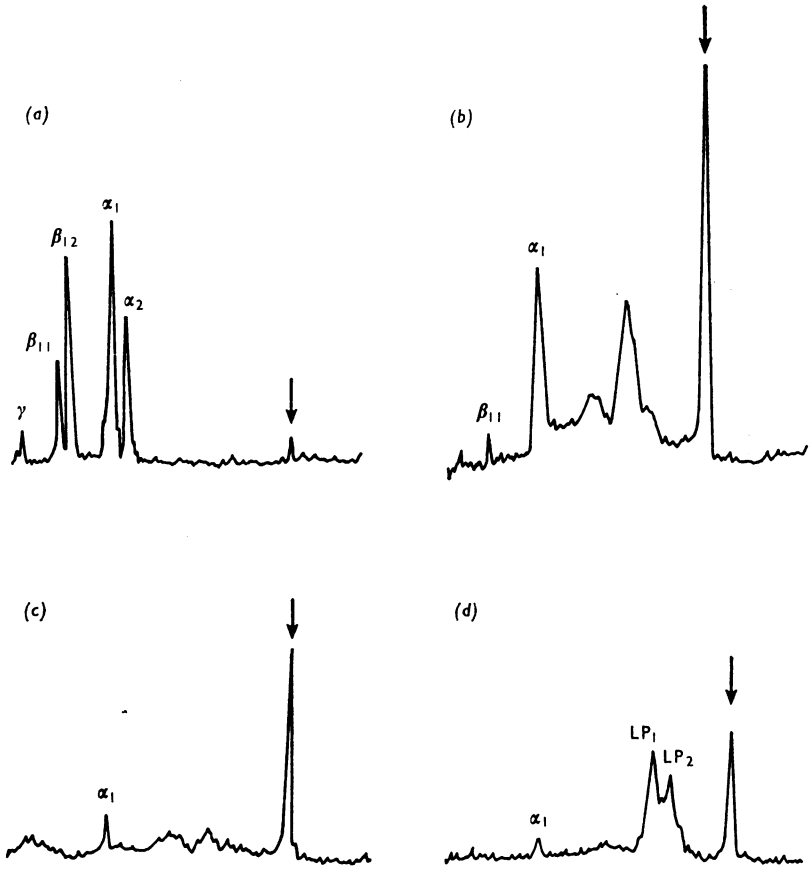


Fig. 5. *Sodium dodecyl sulphate/polyacrylamide-disc-gel electrophoresis of proteoglycan fractions obtained from* 2.0M-*$CaCl_2$-Seq. extracts of bovine nasal cartilage*

Proteoglycan fractions were isolated as described in the text. Densitometric scans of 7% (w/v) polyacrylamide gels after electrophoresis of various fractions are shown. (*a*) Acid-soluble rat skin collagen; (*b*) $gel_{CaCl_2-Seq.}$; (*c*) $A3_{CaCl_2-Seq.}$; (*d*) $A1D3_{CaCl_2-Seq.}$. α, β and γ refer to the positions in the gel to which various collagen polypeptides migrate. LP_1 and LP_2 refer to the bands given by reduced 'link proteins'. The arrows mark the position of the Bromophenol Blue band.

to denature enzymes than is 2.0M-$CaCl_2$. A1 fractions were prepared from both 0.15M-KCl and sequential guanidine hydrochloride extracts, by maintaining the proteinase inhibitors listed above in inhibitory concentrations throughout the procedure, and performing the centrifugation at 10°C.

Under these conditions A1 fractions from the sequential high-ionic-strength extract had exactly the same appearance on large-pore-gel electrophoresis as did $A1_{CaCl_2}$ and $A1_{CaCl_2-Seq.}$ fractions, reported above. We conclude therefore that high-ionic-strength solutions extract at least two major species of proteoglycan subunit from bovine nasal cartilage, which are separated by the above method and are unlikely to be artifacts.

In contrast, A1 fractions from 0.15M-KCl extracts containing proteinase inhibitors have a different appearance on large-pore-gel electrophoresis from the corresponding uninhibited preparations. They show direct evidence of the presence of aggregates, together with two components whose R_x values closely resemble those of bands (1) and (2) of high-ionic-strength extracts. Thus it seems likely that most, but not all, of the proteoglycan in uninhibited iso-osmotic extracts is partially degraded, and we agree with the proposal (Oegema *et al.*, 1975) that it may originate from an enzymic scission in the middle of the core protein. Extraction at low temperature, but without the addition of proteinase inhibitors, does not seem to prevent this process.

This is consistent with the observation that proteoglycan interaction with hyaluronic acid could not be detected by gel-filtration methods for species extracted with iso-osmotic solutions at 4°C (Hardingham & Muir, 1974). However, since 'uninhibited' $A1D1_{KCl}$ fractions have a lower intrinsic viscosity than $A1_{KCl}$ fractions (Roughley & Mason, 1975) and hyaluronic acid and link proteins can be identified in $A1D2_{KCl}$ and $A1D3_{KCl}$ fractions respectively, we propose that small amounts of native aggregated proteoglycan may be extracted by, and survive in, iso-osmotic solutions, but are not detectable by large-pore-gel electrophoresis. Such aggregates may be those that exist in 'matrix fluid' in the tissue (Pita *et al.*, 1975) or similar to those that can be extracted with 0.4M-guanidine hydrochloride from bovine nasal cartilage (Gregory *et al.*, 1970).

McDevitt & Muir (1971) studied proteoglycans from sequential extracts of pig articular cartilage by large-pore-gel electrophoresis, mobilities of components being expressed as a percentage of the mobility of single-chain chondroitin sulphate from the tissue. Table 1(*b*) shows the mobilities of the bovine fractions relative to bovine chondroitin sulphate and compares them with the results obtained by McDevitt & Muir (1971). The mobility of single-chain glycosaminoglycans is a function of their charge density (McDevitt & Muir, 1971), so migration of chondroitin sulphate from bovine and pig tissues should be the same regardless of any possible variation in chain length.

Proteoglycans extracted with low-ionic-strength solutions from both species have very similar mobilities and, therefore, hydrodynamic size. As discussed above, we consider extracts of this type from bovine cartilage to be composed predominantly of partially degraded proteoglycans. Proteoglycan fragments produced by tryptic proteolysis of pig laryngeal cartilage and bovine nasal cartilage $A1D1_{CaCl_2\text{-}Seq.}$ fraction have identical mobilities (Table 1*b*), suggesting that the native proteoglycans from both species are degraded in a similar manner.

The native proteoglycans extracted by high-ionic-strength solutions from pig articular cartilage have lower mobilities (Table 1*b*) than those from bovine nasal cartilage, and are therefore probably of larger hydrodynamic size. The significance of this finding is not understood, since the proteoglycans of bovine articular cartilage are smaller than those found in bovine nasal cartilage (Rosenberg *et al.*, 1973). Baxter & Muir (1975) found that 'core-protein' preparations from pig laryngeal cartilage have a higher serine content than those from bovine nasal cartilage and that more of these residues were substituted with chondroitin sulphate chains in the former than in the latter. Thus proteoglycans from pig hyaline cartilages may, in general, be larger than those from bovine tissues. A precise correlation between proteoglycan hydrodynamic size and physiological role remains to be established, although it is known that changes in size occur during development of pig articular cartilage (Šimůnek & Muir, 1972).

Hydroxyproline has been detected previously in both low- and high-ionic-strength extracts of bovine nasal cartilage (Rosenberg *et al.*, 1970*a*) and in the A3 fraction obtained from 4M-guanidine hydrochloride extracts of the tissue (Hascall & Sajdera, 1969), but not in the A1 fraction or its subfractions. Similarly, it has been detected in the A2 and A3 fractions from calf nasal cartilage, but not in the A1 fraction (Mashburn & Hoffman, 1971).

Rosenberg *et al.* (1973) found between 0.01 and 0.02% (w/w) hydroxyproline in proteoglycan-aggregate fractions and 0.017% (w/w) in proteoglycan-subunit fractions from bovine articular cartilage. Their data therefore suggest that small amounts of collagen may be associated with proteoglycan rather than with proteoglycan aggregates.

In the experiments reported above for bovine nasal cartilage a small amount of collagen remains associated with the $A1_{CaCl_2\text{-}Seq.}$ fraction, which contains proteoglycan aggregates, whereas most of it separates to the top of the CsCl gradient in the associative centrifugation. Collagen could not be detected in the $A1D1_{CaCl_2\text{-}Seq.}$ fraction, inferring that it is associated with aggregates rather than subunits. Whether the association is due to entanglement or is more specific is not known. The identification of the collagen, by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, as type II in these experiments is tentative. More stringent methods are required for definite identification (Eyre & Muir, 1975).

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