Biochem. J. (1973) **131**, 541–553
Printed in Great Britain

The Composition of Cartilage Proteoglycans

AN INVESTIGATION USING HIGH- AND LOW-IONIC-STRENGTH EXTRACTION PROCEDURES

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(*Received* 21 *August* 1972)

1. A proteoglycan fraction (the proteoglycan subunit fraction) was prepared from extracts, with 0.15M-KCl (low-ionic-strength) and 0.5M-$LaCl_3$, 2.0M-$CaCl_2$ and 4.0M-guanidinium chloride (high-ionic-strength), of bovine nasal cartilage by equilibrium-density-gradient centrifugation, essentially as described by Hascall & Sajdera (1969). 2. The use of different centrifugation times showed that near-equilibrium conditions were reached by 48h for the fractions prepared from the high-ionic-strength extracts. The fraction isolated from the low-ionic-strength extract required a longer centrifugation time to reach equilibrium conditions. 3. The composition of the proteoglycan fractions from the various extracts was compared by analyses of their carbohydrate and amino acid contents. Difference indices were calculated from the amino acid analysis to compare the degree of compositional relationship between the protein components of the proteoglycans. 4. Small compositional differences were found between the proteoglycans isolated from the various high-ionic-strength extracts. The protein content of the fractions from the $CaCl_2$ extract and the guanidinium chloride extract showed the greatest difference in this respect, although their amino acid analysis was similar. 5. The proteoglycan fraction isolated from the low-ionic-strength extract shows marked differences in composition from the fractions isolated from the high-ionic-strength extracts. Its protein and glucosamine contents were lower whereas its hexuronic acid and galactosamine contents were higher than those of the latter. It also exhibits major differences in its amino acid composition. The glucosamine:galactosamine ratio of the fraction from the low-ionic-strength extract indicates that it may be an almost exclusively chondroitin sulphate–proteoglycan. Its analysis correlates closely with that of a low-molecular-weight proteoglycan isolated from pig laryngeal cartilage by Tsiganos & Muir (1969). 6. The proteoglycan fractions from both the low- and high-ionic-strength extracts migrate as a single band in zone electrophoresis carried out in a sucrose-density gradient at both pH3.0 and pH7.0, although each showed evidence of band widening during the electrophoresis. All the proteoglycan fractions migrated with the same electrophoretic mobility at pH3.0, irrespective of the differences in composition between them. 7. The differences between the proteoglycans from the low- and high-ionic-strength extracts are discussed and the view is advanced that they may be due to association between predominantly chondroitin sulphate–proteoglycans and a keratan sulphate-enriched proteoglycan species.

Extracts of cartilage protein–polysaccharides can be resolved into a number of different fractions of different protein and carbohydrate content by several methods. A preparation (protein–polysaccharide light fraction) obtained from bovine nasal cartilage, for example, can be further resolved into four fractions of differing composition by ultracentrifugation in the presence of univalent and bivalent cations (Pal *et al.*, 1966). Tsiganos & Muir (1969) showed that material extracted from pig laryngeal cartilage with iso-osmotic neutral (0.15M) sodium acetate and neutral 10% (w/v) $CaCl_2$ could be fractionated by chromatography on agarose gels or by zone electrophoresis into components with different antigenic determinants as well as different chemical compositions.

From these and other examples it is apparent that protein–polysaccharides exist in the cartilage as a heterogeneous population of molecules. Schubert (1966) suggested that this heterogeneity may result from the presence of a discrete series of a small number of different protein–polysaccharides rather than a continuous polydisperse series of such compounds.

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Other investigators, however, have proposed that, since even mild methods of extraction yield a population of protein–polysaccharides with an extensive degree of heterogeneity, the definition of a discreet protein–polysaccharide molecule is not possible (Mashburn *et al.*, 1965).

The application of equilibrium isopycnic density-gradient centrifugation to the fractionation of protein–polysaccharide preparations has shown that a protein-rich fraction containing very little carbohydrate can be separated from the much denser, carbohydrate-rich, protein–polysaccharide component (Franek & Dunstone, 1967). Sajdera & Hascall (1969) utilized this principle to isolate the dense protein–polysaccharide component of extracts of bovine nasal cartilage made with salt solutions of high ionic strength. Subsequent equilibium-density-gradient centrifugation of the dense protein–polysaccharide in the presence of 4.0 M-guanidinium chloride enabled it to be resolved from further protein components, which were recovered in the upper part of the gradient. The protein–polysaccharide that sedimented to the densest part of this second gradient should, theoretically, represent a macromolecular species separated from both unbound and ionically associated protein. It has been termed the proteoglycan subunit fraction (Hascall & Sajdera, 1969).

Proteoglycan subunit isolated from 4.0M-guanidinium extracts of bovine nasal cartilage has been shown to be polydisperse with respect to both its molecular weight and chemical composition (Hascall & Sajdera, 1970). The experiments reported below were undertaken to ascertain whether the composition of bovine nasal cartilage proteoglycan subunit was in any way dependent on the nature of the salt solution used to extract the cartilage. Proteoglycan subunit has been isolated from a variety of low- and high-ionic-strength salt extracts of the tissue and the composition of the fractions has been compared. Prolonged centrifugation times have been used to approach true equilibrium conditions for the isolation of proteoglycan subunit.

Preliminary reports of some of this work have been made (Mason & Mayes, 1972, 1973*a*).

Experimental

Extraction of protein–polysaccharide from cartilage slices

Methods for the extraction of protein–polysaccharide from fresh bovine nasal cartilage of 2-year-old animals with unbuffered inorganic salt solutions have been described in the preceding paper (Mason & Mayes, 1973*b*). The salt solutions used for the extraction were 0.15M-KCl (*I* 0.15), 0.5M-$LaCl_3$ (*I* 3.0) and 2.0M-$CaCl_2$ (*I* 6.0). The lowest-ionic-strength solution extracts approx. 18%, and the other two higher-ionic-strength solutions 80%, of the total protein–polysaccharide present in the tissue (Mason & Mayes, 1973*b*).

Extraction of cartilage slices with 4.0M-guanidinium chloride (*I* 4.0) was carried out in the same manner as described for inorganic salt solutions except that the extracting solution was buffered with sodium acetate buffer, pH5.8, which was 0.05M in acetate ions (Hascall & Sajdera, 1969). Guanidinium chloride (BDH Chemicals Ltd., Poole, Dorset, U.K.) was recrystallized three times from ethanol before use. A 4.0M solution of the salt extracts 80% of the total protein–polysaccharide present in the tissue (Sajdera & Hascall, 1969).

Fractionation of the cartilage extract

Filtrates of the cartilage extract (see Mason & Mayes, 1973*b*) other than those obtained from the $LaCl_3$ extractions (see below) were dialysed against water (100vol.) to remove excess of salt. The volumes in each case were then halved by rotary evaporation under reduced pressure at 30°C. Sodium acetate was added to the solution to a concentration of 0.05M and the pH was adjusted to 5.8 where necessary. Solid CsCl was added to the buffered solution to give a density of 1.69g/ml (Sajdera & Hascall, 1969).

The solution was then fractionated by the density-gradient-centrifugation method of Sajdera & Hascall (1969) with the following differences. Centrifugation was carried out for 48h at 20°C with the MSE Superspeed 65 Mk II centrifuge. The rotor (MSE no. 59594, 8×25ml capacity) was operated at 83850*g* ($r_{av.}$ = 6.887cm). At the end of the centrifugation the density gradient was fractionated by piercing the bottom of the centrifuge tube and collecting fractions. Fractions from any one preparation with densities greater than 1.70g/ml were pooled, dialysed against water and then desalted on a Sephadex G-25 column (Mason & Wusteman, 1970). The salt-free protein–polysaccharide preparation was freeze-dried.

The proteoglycan subunit fraction described by Hascall & Sajdera (1969) was prepared from the freeze-dried protein–polysaccharide by a second density-gradient centrifugation. The composition of the initial solution was the same as that reported by Hascall & Sajdera (1969), but the centrifugation was carried out with the MSE Superspeed Mk II centrifuge as described above. Centrifugation was carried out for periods of 48, 60, 72 or 96h for each protein–polysaccharide preparation. The final density gradient was fractionated in the same manner as for the preparation of protein–polysaccharide. Fractions with densities greater than 1.55g/ml were pooled, dialysed against water, desalted on a Sephadex G-25 column and freeze-dried. The freeze-

dried preparation corresponds to the proteoglycan subunit preparation of Hascall & Sajdera (1969).

Special treatment of extracts made with $LaCl_3$

The filtrates obtained from $LaCl_3$ extractions were diluted with 9vol. of water and left for 1h at 4°C to precipitate the lanthanum salt of the protein–polysaccharide (Mason & Mayes, 1973*b*). The precipitate was collected by centrifuging and dissolved in sodium EDTA (8.18g of EDTA/litre, 3.36g of NaOH/litre, pH5.8) as described by Doganges & Schubert (1964). Potassium acetate was added to the solution (final concn. 5%, w/v). The potassium salt of the protein–polysaccharide was then precipitated by the addition of ethanol (3vol.). The mixture was left overnight at 4°C and then the precipitate was collected by centrifuging, washed with ethanol (80%, v/v) and finally dissolved in water. After dialysis of the solution against water (100vol.) the fractionation procedure described above was followed to obtain first the purified protein–polysaccharide and then the proteoglycan subunit fraction.

Zone electrophoresis in a sucrose-density gradient

Analytical zone electrophoresis of proteoglycan subunit preparations was carried out in a sucrose-density gradient. The electrophoresis column (ISCO model no. 210; Instrumentation Specialities Co. Ltd., Lincoln, Nebr., U.S.A.) and accessory equipment were described in detail by Brakke *et al.* (1968). The apparatus is designed to allow observation of components moving through the gradient during electrophoresis by pumping the gradient past a u.v. (255nm) optical unit at predetermined time-intervals. Thus the electrophoresis of a single-component sample, or the separation of a sample into more than one component, can be followed on the column. The electrophoretic mobility, m, of a component is given by:

$$m = \frac{ds}{dt} \cdot \frac{k\pi r^2}{I} \qquad (1)$$

where I is the current passed, k is the conductivity of the buffer and r is the radius of the column, which in the present experiments was 0.5cm. The observed mobility of the component during electrophoresis is expressed by ds/dt, where s is the distance moved by the component from its initial position in time t.

In the present experiments ds/dt was calculated by making a number of measurements of s at 900s intervals during the electrophoresis. The best value for ds/dt was determined by the method of least-squares regression (see Figs. 3 and 4).

The solutions for the sucrose-density gradient were prepared as follows. The exact concentration of a stock solution of sucrose in water (approx. 50%, w/v) was determined by refractometry (Abbé Refractometer model B; Carl Zeiss, Oberkochen, Germany). All solutions were then prepared from the stock sucrose solution and a sodium citrate buffer (1M in citrate), pH3.00. The bottom of the electrophoresis column and adjacent electrode chamber (cathode) contained 40% (w/v) sucrose–0.02M-sodium citrate, pH3.00. A density gradient of 35–10% (w/v) sucrose buffered with 0.02M-sodium citrate, pH3.00, was prepared from 35% (w/v) buffered sucrose (4.5ml) and 10% (w/v) buffered sucrose (4.5ml) by using an MSE gradient maker (cat. no. GA 100) and was pumped on top of the 40% (w/v) sucrose.

The sample (approx. 1.0mg) in 0.1ml was dissolved in 5% (w/v) sucrose buffered with 0.02M-sodium citrate, pH3.00, and left overnight at 4°C. The sample was centrifuged in a bench centrifuge for 15min before being layered on top of the density gradient in the electrophoresis column. Sodium citrate buffer (0.02M, pH3.00) was then layered on top of the sample to fill the top of the column and maintain electrical contact with the top electrode chamber (anode), which also contained the same buffer.

The electrophoretic properties of samples were also examined at pH7.0. In this case a 0.01M-potassium phosphate buffer was used to maintain pH. At both pH3.0 and pH7.0 the electrophoresis was carried out at 4°C and a constant current of 5mA was applied. The voltage was approx. 900V when electrophoresis was performed with the density gradient in the elevated position in the column (see Brakke *et al.*, 1968) and 500V when it was in the lower position.

Analytical methods

Hexuronic acid and hexose. The method of Bitter & Muir (1962) was used for hexuronic acid with D-glucuronolactone as standard. Hexose was assayed by the anthrone reaction (Dische, 1962), with D-galactose as standard.

Hexosamine. Glucosamine and galactosamine were assayed by using the Locarte amino acid analyser. The 15cm×0.9cm column was filled with resin to a height of 13cm and eluted with the standard 0.35M-sodium citrate buffer, pH5.28. The column was operated at 60°C. Protein–polysaccharide preparations were hydrolysed before analysis and corrections applied for losses of hexosamine during hydrolysis as described previously (Mason & Wusteman, 1970).

Amino acid analysis. The amino acid analysis of protein–polysaccharide preparations was performed by using a single-column technique with a Locarte amino acid analyser. Samples were hydrolysed (110°C for 24h in 6M-HCl) *in vacuo*. Hydrolysates were then

evaporated to dryness in a desiccator at 40°C. The dry residue was redissolved in a pyridine–acetic acid buffer, pH6.5 (pyridine–acetic acid–water, 25:1:474, by vol.) and left in air for 4h at room temperature to oxidize cysteine to cystine (Landon *et al.*, 1971). Hydrolysates were then evaporated to dryness in the desiccator and the dry residue was re-dissolved in 0.2M-sodium citrate buffer, pH2.2. Amino acid analysis was performed on the 30cm column of the Locarte analyser with a stepwise gradient of sodium citrate buffers, pH3.25 (33min), pH4.48 (110min) and pH6.65 (150min). The column was operated at 50°C for the first buffer and at 60°C thereafter. With this system glucosamine and galactosamine emerge after phenylalanine, well separated from one another and from the basic amino acids. A number of minor ninhydrin-reactive components present in protein–polysaccharide hydrolysates, assumed to be the degradation products of hexosamines, are also resolved from histidine and lysine by using this buffer sequence.

Protein. The protein content of protein–polysaccharide preparations was calculated from the amino acid analysis. No corrections were applied for amino acid losses during hydrolysis.

Proteoglycan subunit preparations were dried to a constant weight at 70°C *in vacuo* over P_2O_5 before analysis of their composition.

Results

Equilibrium-density-gradient centrifugation of cartilage extracts

Cartilage extracts prepared with 0.15M-KCl, 0.5M-$LaCl_3$, 4.0M-guanidinium chloride and 2.0M-$CaCl_2$ were centrifuged for 48h in CsCl as described in the Experimental section. Fractionation of the centrifuge tube at the end of the run and analysis of the fractions for hexuronate and protein indicates that a dense protein–polysaccharide sediments to the bottom of the tube and a protein fraction is recovered from the top of the tube. A typical analysis is shown for a cartilage extract prepared with $LaCl_3$ in Fig. 1. In addition a gel-like material is found floating on the top of the gradient at 48h for each of the extracts made with the solutions listed above. These results are similar to those reported by Sajdera & Hascall (1969) and Hascall & Sajdera (1969) for cartilage extracts in $MgCl_2$ and guanidinium chloride respectively. However, the proportion of the total hexuronic acid recovered in the fractions

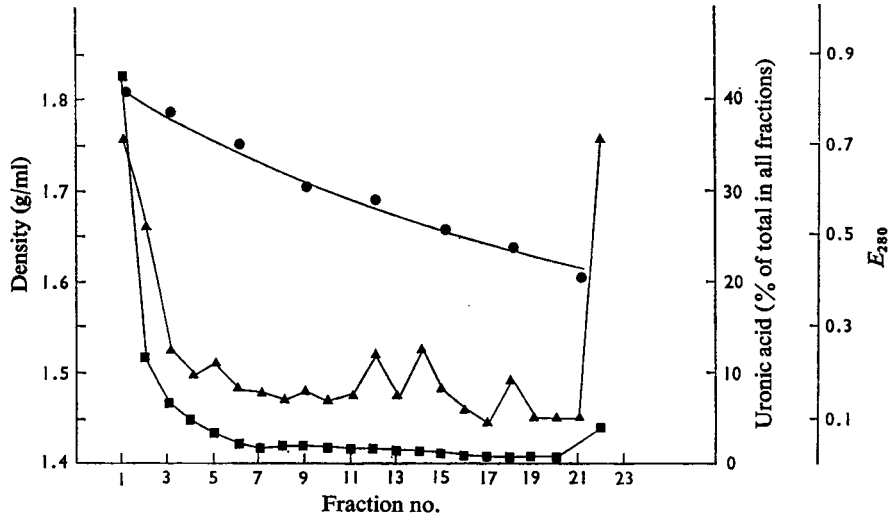

Fig. 1. *Equilibrium-density-gradient centrifugation of a $LaCl_3$ extract of bovine nasal cartilage*

Bovine nasal cartilage was extracted with 0.5M-$LaCl_3$. After conversion of the extracted material into the potassium salt, it was centrifuged for 48h in CsCl (see the Experimental section for details). After centrifugation the bottom of the tube was pierced and fractions (0.8ml) were collected and analysed. ●, Density (g/ml); ▲, E_{280}; ■, percentage of the total hexuronic acid in the centrifuge tube recovered in each fraction. Fractions were diluted 1:4 (v/v) with water for the measurement of E_{280} or 1:40 (v/v) for the determination of hexuronic acid. An insoluble gel which collected on top of the gradient during centrifugation is not represented in the figure.

with $\rho > 1.70$ was always about 65.0% in our experiments, whereas Hascall & Sajdera (1969) report a value of 95%.

Equilibrium-density-gradient centrifugation of protein–polysaccharide

The protein–polysaccharide isolated from each of the different salt extractions and recovered in the fractions having a density greater than 1.70g/ml (see above) was further fractionated by equilibrium-density-gradient centrifugation in the presence of 4.0M-guanidinium chloride (see the Experimental section for details). At the end of the centrifugation the tubes were pierced and fractions were collected and analysed as described above. A typical analysis is shown in Fig. 2.

Approximately 85% of the total hexuronate content of each tube was found in the fractions having a density greater than 1.55g/ml, and these fractions were pooled and designated proteoglycan subunit fraction, in accordance with the nomenclature of Hascall & Sajdera (1969). These authors reported a recovery of 95% of the total hexuronate in the centrifuge tube in the proteoglycan subunit fraction prepared from a 4.0M-guanidinium cartilage extract (Hascall & Sajdera, 1969).

In our experiments, although there was a concentration of protein-containing material in the fractions having a density greater than 1.55g/ml, as judged by the extinction at 280nm, protein was also dispersed throughout the remainder of the centrifuge tube. Only a marginal increase in E_{280} was noted for fractions having the lowest density (see Fig. 2).

Composition of the proteoglycan subunit fraction

The effect of two different factors on the composition of the proteoglycan subunit fraction was studied. These were (*a*) the influence of the nature of the salt solution used to prepare cartilage extracts, from which protein-polysaccharide and subsequently proteoglycan subunit fraction was isolated, and (*b*) the effect of prolonged centrifugation time of protein–polysaccharide in the presence of 4.0M-guanidinium chloride on the composition of the proteoglycan subunit fraction.

Protein–polysaccharide was isolated from 0.15M-KCl, 0.5M-$LaCl_3$, 4.0M-guanidinium chloride and 2.0M-$CaCl_2$ cartilage extracts as described above. Identical weights of each protein–polysaccharide preparation were then centrifuged for 48, 60, 72 or 96h as described in the Experimental section. The overall compositions and amino acid analyses of the proteoglycan subunit fractions recovered from each of these experiments are reported in Tables 1 and 2 respectively.

Extension of the centrifugation time of protein–polysaccharide obtained from each of the high-ionic-strength cartilage extracts beyond 48h appears to have little effect on the composition of the proteoglycan subunit fraction subsequently isolated (Tables 1 and 2). Minor differences in the composition of the proteoglycan subunit fractions isolated from the

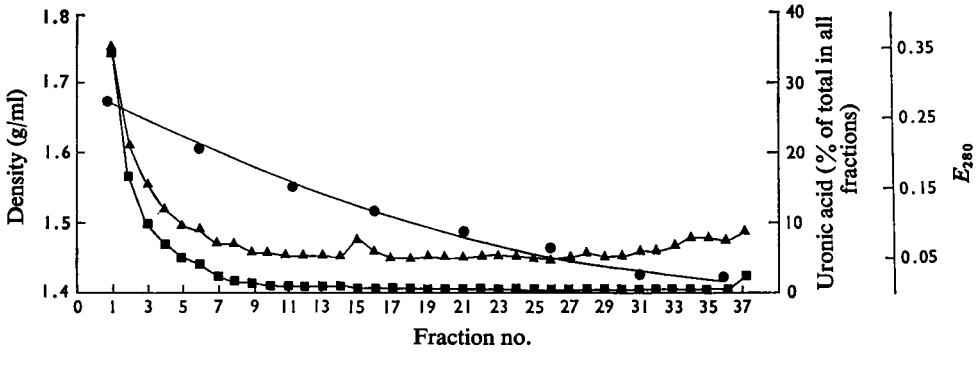

Fig. 2. *Equilibrium-density-gradient centrifugation of protein–polysaccharide isolated from an $LaCl_3$ extract of bovine nasal cartilage*

Protein–polysaccharide isolated from a 0.5M-$LaCl_3$ extract of the cartilage by equilibrium-density-gradient centrifugation (see the Experimental section and Fig. 1) was re-centrifuged for 96h in CsCl containing 4.0M-guanidinium chloride. Details are given in the Experimental section. After centrifugation the bottom of the tube was pierced and fractions (0.5ml) were collected and analysed. ●, Density (g/ml); ▲, E_{280}; ■, percentage of the total hexuronic acid in the centrifuge tube recovered in each fraction. Fractions were diluted 1:4 (v/v) with water for the measurement of E_{280} or 1:40 (v/v) for the estimation of hexuronic acid.

Table 1. *Composition of proteoglycan subunit fraction isolated from different salt extractions of bovine nasal cartilage*

Protein–polysaccharide was isolated from 0.15M-KCl, 0.5M-$LaCl_3$, 4.0M-guanidinium chloride and 2.0M-$CaCl_2$ cartilage extracts as described in the text. The proteoglycan subunit fraction was then isolated from each protein–polysaccharide preparation by equilibrium-density-gradient centrifugation in CsCl and 4.0M-guanidinium chloride. Centrifugation times of 48, 60, 72 or 96h were used for the protein–polysaccharide preparations (see the Experimental section for details). Each value given is the mean for two separate analyses.

	Composition of proteoglycan subunit (g/100g of proteoglycan)											
	0.15M-KCl extract			0.5M-$LaCl_3$ extract			4.0M-Guanidinium chloride extract			2.0M-$CaCl_2$ extract		
Centrifugation time (h) ...	48	60	96	48	72	96	48	72	96	48	60	96
Galactosamine (free base)	28.8	27.5	27.0	19.1	20.8	22.0	22.1	21.3	19.3	20.0	20.2	21.8
Glucosamine (free base)	0.4	0.5	0.6	1.8	1.9	2.0	2.3	2.2	2.1	1.8	1.5	1.7
Glucuronic acid (lactone)	29.3	26.9	26.0	19.7	21.5	22.1	21.0	20.5	21.2	19.1	22.1	22.8
Protein	3.9	3.3	3.6	5.4	6.7	6.3	6.6	7.2	8.0	4.6	5.2	5.4
Galactosamine:glucuronic acid molar ratio	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.9	1.0	0.9	0.9
Galactosamine:glucosamine molar ratio	73.9	51.4	47.9	10.9	10.9	11.1	9.8	9.7	9.1	11.4	13.7	13.2

Table 2. *Amino acid composition of proteoglycan subunit fraction isolated from different salt extractions of bovine nasal cartilage*

Protein–polysaccharide was isolated from 0.15M-KCl, 0.5M-$LaCl_3$, 4.0M-guanidinium chloride and 2.0M-$CaCl_2$ cartilage extracts as described in the text. The proteoglycan subunit fraction was then isolated from each protein–polysaccharide preparation by equilibrium-density-gradient centrifugation in CsCl and 4.0M-guanidinium chloride. Centrifugation times of 48, 60, 72 or 96h were used for the protein–polysaccharide preparations (see the Experimental section for details). Each value given is the mean of two separate analyses. No corrections were applied for amino acid losses during hydrolysis. n.d., Not detected; tr, trace.

	Amino acid composition of proteoglycan subunit (mol/1000mol of amino acid residues)											
	0.15M-KCl extract			0.5M-$LaCl_3$ extract			4.0M-Guanidinium chloride extract			2.0M-$CaCl_2$ extract		
Centrifugation time (h) ...	48	60	96	48	72	96	48	72	96	48	60	96
Aspartic acid	57.3	59.3	59.7	74.2	69.5	68.7	67.7	69.2	68.7	63.4	65.8	66.4
Threonine	43.1	44.7	46.2	61.0	58.1	57.9	58.5	59.9	61.5	54.7	61.5	61.3
Serine	162.2	157.3	154.4	116.1	109.6	110.2	107.0	111.6	107.9	119.7	122.1	119.9
Glutamic acid	148.8	147.4	147.1	147.8	150.1	148.1	149.5	147.4	153.3	150.3	147.2	150.9
Proline	81.8	91.6	95.6	118.7	110.1	107.8	112.1	104.9	101.3	105.7	115.5	114.5
Glycine	165.2	149.5	148.0	104.9	108.0	112.3	109.5	118.1	111.8	117.0	120.1	115.1
Alanine	57.9	61.7	62.1	68.7	73.1	73.0	73.1	72.3	73.3	73.1	68.7	71.6
Cystine (half)	n.d.	n.d.	n.d.	tr	tr	tr	tr	tr	tr	tr	n.d.	n.d.
Valine	71.0	75.0	75.7	65.3	70.2	68.8	69.4	67.3	66.8	75.2	65.3	65.4
Methionine	tr	tr	tr	(~4.0)	(~4.0)	(~4.0)	(~4.0)	tr	(~4.0)	(~4.0)	(~4.0)	(~4.0)
Isoleucine	41.0	40.0	39.0	36.6	36.9	38.1	37.2	40.1	37.6	37.6	35.7	37.9
Leucine	94.3	90.2	87.9	77.7	78.5	79.9	80.1	80.7	81.6	79.5	77.1	76.5
Tyrosine	6.5	7.4	7.5	18.4	21.1	21.3	20.1	18.8	22.1	19.0	17.2	17.4
Phenylalanine	24.5	25.4	25.9	34.3	34.0	36.6	37.8	36.6	37.1	33.8	33.4	33.6
Histidine	14.4	15.3	15.3	14.2	14.7	15.0	14.4	14.1	14.5	13.9	13.7	13.6
Lysine	13.6	13.5	13.3	25.3	26.6	23.7	22.0	21.9	23.5	19.3	21.2	21.8
Arginine	19.4	21.8	22.3	36.9	38.7	38.7	40.4	37.1	39.3	37.9	35.7	34.8

same high-ionic-strength cartilage extract may in part be due to small variations attributable to the analytical procedures used.

In contrast with the above, prolonged centrifugation of protein–polysaccharide obtained from the 0.15M-KCl cartilage extract yields a proteoglycan subunit fraction having a glucosamine:galactosamine ratio different from that obtained with a 48h centrifugation (Table 1). Some differences are also discernible in the amino acid composition of proteoglycan subunit fractions from the 48, 60 and 96h centrifugations of protein–polysaccharide from the 0.15M-KCl cartilage extract. The proline content is increased and the glycine content decreased, each by over 10 residues/1000 residues, in proteoglycan fractions isolated from the extended centrifugations (Table 2).

One method of comparing the difference between the amino acid composition of two proteins is by computing a difference index for them (Metzger *et al.*, 1968). The difference in the fractional contents of each amino acid is found for the two proteins and the sum of the absolute value of those differences is multiplied by 50 to give the difference index. Two proteins with no amino acid in common have a difference index of 100. Two proteins with the same composition have a difference index of 0 (Metzger *et al.*, 1968). The amino acid compositions of the proteoglycan subunit fractions isolated from protein–polysaccharide obtained from a particular extraction were compared in this way, the difference index being calculated between the 48h fraction and the 72h and 96h fractions. The results are shown in

Table 3. *Comparison, by difference index, of the amino acid composition of proteoglycan subunit fractions isolated from protein–polysaccharide preparations after various centrifugation times*

The proteoglycan subunit fraction was isolated from protein–polysaccharide preparations obtained from various salt extracts of bovine nasal cartilage by equilibrium-density-gradient centrifugation for 48, 60, 72 or 96h (see the Experimental section). The amino acid composition of the fractions is shown in Table 2. Difference indices were calculated for the fraction from the 48h centrifugation and proteoglycan subunit recovered from the prolonged centrifugations. The index is an estimate of the overall difference between the amino acid compositions of the proteoglycan subunit fractions (see the text).

Protein–polysaccharide source of proteoglycan subunit ...	KCl extract		$LaCl_3$ extract		Guanidinium chloride extract		$CaCl_2$ extract	
Centrifugation time (h) ...	60	96	72	96	72	96	60	96
Difference index, compared with 48h centrifugation proteoglycan subunit	2.62	3.44	2.25	2.69	1.95	1.71	2.15	2.26

Table 4. *Statistical analysis of the protein composition of proteoglycan subunit fractions isolated from protein–polysaccharide obtained from different salt extractions of bovine nasal cartilage*

Protein–polysaccharide was prepared from 0.15M-KCl, 0.5M-$LaCl_3$, 4.0M-guanidinium chloride and 2.0M-$CaCl_2$ cartilage extracts. Proteoglycan subunit fraction was isolated from each protein–polysaccharide preparation by equilibrium-density-gradient centrifugation for 48, 60, 72 or 96h (see the Experimental section). The protein content of each proteoglycan subunit fraction was calculated from its amino acid analysis. The amino acid analysis of proteoglycans originating from the same cartilage extract but prepared after different centrifugation times was similar (see the text). A mean and standard deviation (S.D.) was therefore calculated for the protein content of such proteoglycans. Six analyses were performed with each extract. A two-sample Student's *t* test was used to compare the mean protein contents of proteoglycans isolated from the 0.15M-KCl cartilage extract with those of proteoglycans isolated from high-ionic-strength extracts.

Protein–polysaccharide source of proteoglycan subunit ...	KCl extract	$LaCl_3$ extract	Guanidinium chloride extract	$CaCl_2$ extract
Mean protein content (g/100g of proteoglycan)	3.60	6.13	7.25	5.08
S.D.	0.342	1.118	0.717	0.525
t	—	−5.769	−5.291	−11.246
P	—	<0.001	<0.001	<0.001

Table 3. All the difference indices are very small, the largest being that for the 48h and 96h preparations of proteoglycan subunit from the protein–polysaccharide of low-ionic-strength extract. This is in contrast to the difference indices found between proteoglycan subunit fractions originating from different salt extractions of the cartilage (see below).

A comparison of the composition of proteoglycan subunit fractions isolated from the four different salt extracts of the cartilage indicates that distinct differences occur between them. The protein content of proteoglycan subunit originating from any one type of cartilage extract appears to be characteristic for that proteoglycan. Since the difference indices for the amino acid composition of proteoglycans isolated after 48, 60, 72 or 96h centrifugation of protein–polysaccharide from any one particular cartilage extract were small (Table 3), the analyses for the protein content of each proteoglycan were combined and expressed statistically (Table 4). The results show that the protein content of the proteoglycan subunit fraction isolated from the 0.15M-KCl extract is significantly lower ($P<0.001$) than that of proteoglycan subunit fractions isolated from the high-ionic-strength cartilage extracts. There is some variation in the protein content of the latter proteoglycans, that from the 4.0M-guanidinium chloride extraction having the highest protein content.

The proteoglycan subunit fraction from the 0.15M-KCl extract also shows other differences in composition compared with the proteoglycans isolated from the high-ionic-strength extracts. The galactosamine and hexuronic acid contents are higher and the glucosamine content is markedly lower than those of the high-ionic-strength extract proteoglycans (Table 1). These features indicate that the low-ionic-strength extract proteoglycan is enriched in chondroitin sulphate but lower in keratan sulphate content than proteoglycans isolated from the high-ionic-strength extractions (see Mason, 1970).

A further difference in the composition of the proteoglycan subunit fractions from low- and high-ionic-strength extracts is apparent in their amino acid composition. Proteoglycan from the low-ionic-strength extract is considerably enriched in its serine and glycine content, but has a lower proportion of aromatic and basic amino acids than proteoglycans isolated from the high-ionic-strength extractions (Table 2). A higher serine content may be expected in a proteoglycan relatively enriched in chondroitin sulphate, since this amino acid residue provides the linkage point for the attachment of the glycosaminoglycan to the protein core of the molecule (Rodén & Smith, 1966).

Difference indices were calculated from the amino acid analyses of the proteoglycans isolated from the different salt extractions of the cartilage, by using the method described above. The indices further

Table 5. *Comparison, by difference index, of the amino acid composition of proteoglycan subunit fractions isolated from extracts of bovine nasal cartilage made with different salt solutions*

Proteoglycan subunit fractions were isolated from protein–polysaccharide preparations obtained from 0.15M-KCl, 0.5M-$LaCl_3$, 4.0M-guanidinium chloride and 2.0M-$CaCl_2$ extracts of bovine nasal cartilage (see the Experimental section). Difference indices were calculated from the amino acid composition of the proteoglycan subunit fractions as described in the text. The index is an estimate of the overall difference between amino acid compositions of the proteoglycan subunit fractions (see the text). Difference indices were calculated for proteoglycan subunit fractions isolated after 48 and 96h equilibrium-density-gradient centrifugation of protein–polysaccharide.

Protein–polysaccharide source of proteoglycan subunit fraction	Protein–polysaccharide preparation ...	48h			96h		
		$CaCl_2$ extract	$LaCl_3$ extract	Guanidinium chloride extract	$CaCl_2$ extract	$LaCl_3$ extract	Guanidinium chloride extract
KCl extract		10.85	13.32	20.38	8.69	10.11	10.02
$CaCl_2$ extract		—	3.68	3.28	—	2.57	2.90
$LaCl_3$ extract		—	—	3.28	—	—	1.42

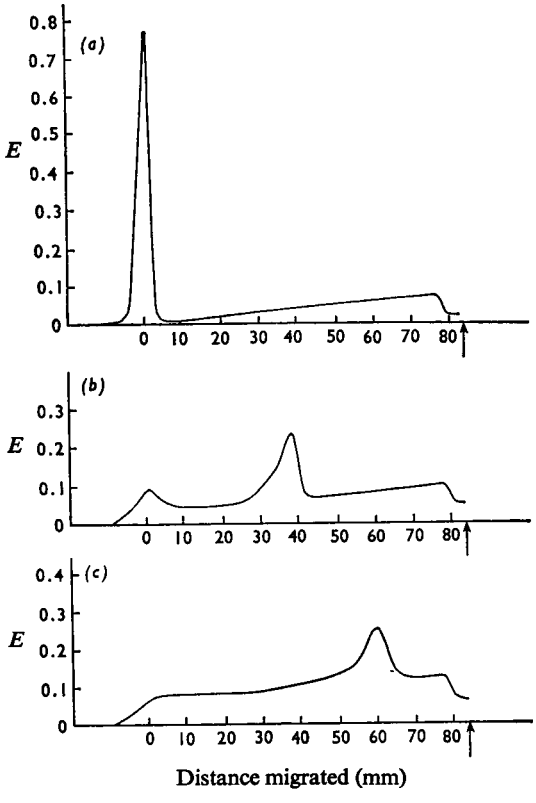

Fig. 3. *Zone electrophoresis of a proteoglycan subunit fraction isolated from a* 2.0M-$CaCl_2$ *extract of bovine nasal cartilage*

The proteoglycan subunit fraction was isolated from protein–polysaccharide obtained from a 2.0M-$CaCl_2$ extract of the cartilage after centrifugation for 48h in the presence of 4.0M-guanidinium chloride (see the Experimental section). Electrophoresis of the proteoglycan fraction was carried out at pH3.0 in a sucrose density gradient as described in the Experimental section. The design of the electrophoresis column allows the gradient to be scanned at 255nm before and during the electrophoresis. Three such scans of the gradient are shown: (*a*) before electrophoresis, (*b*) after 60min electrophoresis and (*c*) after 120min electrophoresis. The ordinate is calibrated in absorbance units. The distance moved by a component in the gradient after a given time of electrophoresis can be measured as the distance (*s*) between the peak maxima of the component and a pre-set calibration mark (↑) on the abscissa. The small absorbance peak at the top of the gradient (anode) in the 60min scan is due to a meniscus at the point where the proteoglycan sample was layered on. This has largely disappeared in the 120min scan.

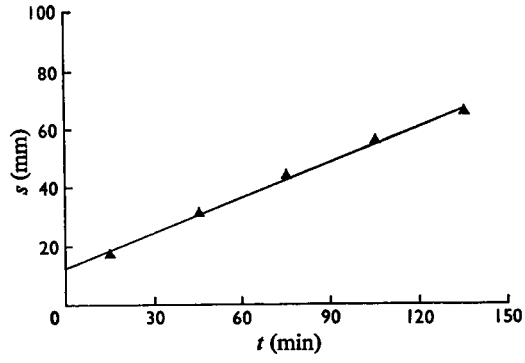

Fig. 4. *Plot of the distance moved by a proteoglycan subunit fraction in time t during zone electrophoresis in a sucrose density gradient*

Zone electrophoresis was carried out at pH3.0 as described in the Experimental section. The results presented are for the electrophoresis of the proteoglycan fraction shown in Fig. 3. A series of values have been plotted for the distance (*s*) moved by the proteoglycan after *t* min of electrophoresis. The value of *s* is accurately measured from a chart record of the absorbance scan of the sucrose density gradient (see Fig. 3). The electrophoretic mobility (*m*) of the proteoglycan can be determined from the slope of the line d*s*/d*t* (see the Experimental section).

demonstrate the degree of compositional difference between the protein component of the proteoglycan subunit fraction isolated from the low-ionic-strength extract and the protein of the corresponding fractions from the high-ionic-strength extracts (Table 5). Difference indices for the proteoglycans isolated from each of the three high-ionic-strength extractions are small, confirming that they are closely related in their protein composition but not identical (Tables 2, 4 and 5). All the difference indices are decreased in proteoglycans recovered from the 96h equilibrium-density-gradient centrifugation of the respective protein–polysaccharide preparations, compared with those from the 48h centrifugations.

Zone electrophoresis of the proteoglycan subunit fractions

Electrophoresis of the proteoglycan subunit fractions isolated from the low- and high-ionic-strength salt extracts of the cartilage was carried out in a sucrose density gradient as described in the Experimental section. The method enables the movement of the molecule to be recorded at predetermined intervals during the electrophoresis. A typical series of

Table 6. *Electrophoretic mobility of proteoglycan subunit fractions isolated from extracts of bovine nasal cartilage made with different salt solutions*

Proteoglycan subunit fractions were isolated from protein–polysaccharide preparations obtained from 0.15M-KCl, 0.5M-$LaCl_3$, 4.0M-guanidinium chloride and 2.0M-$CaCl_2$ extracts of bovine nasal cartilage. A 48h centrifugation was used to prepare the proteoglycans (see the Experimental Section). Zone electrophoresis of the proteoglycan fractions was carried out in a sucrose density gradient at pH3.0 and the electrophoretic mobility (m) of the fractions was determined as described in the Experimental section (see also Figs. 5 and 6).

Protein–polysaccharide source subunit fraction	$10^{10} \times$ Electrophoretic mobility, m ($m^3 \cdot s^{-1} \cdot V^{-1}$)
KCl extract	0.482
$LaCl_3$ extract	0.493
Guanidinium chloride extract	0.472
$CaCl_2$ extract	0.504

scans of the gradient during the electrophoresis of a proteoglycan subunit fraction is shown in Fig. 3.

All the proteoglycans examined were obtained from 48h equilibrium-density-gradient centrifugations of the respective protein–polysaccharides. The electrophoretic properties of the proteoglycans originating from both the low- and high-ionic-strength extracts were similar. Each moved through the sucrose density gradient as a single component at both pH3.0 and pH7.0. During the electrophoresis, the originally narrow absorbance peak of each proteoglycan fraction showed considerable broadening (Fig. 3).

The electrophoretic mobility of each proteoglycan fraction was determined from measurements made at set time-intervals (t) of the distance (s) moved by the molecule in the electric field (see the Experimental section and Fig. 3). A plot of a series of measurements for s is shown in Fig. 4. The mobilities of the proteoglycan fractions at pH3.0 are given in Table 6 and are very similar for the four different preparations.

Discussion

Protein–polysaccharides are a major component of the extracellular matrix of hyaline cartilage. The study of the physicochemical properties of these molecules and the nature of their association with other components of the matrix such as collagen and non-fibrous proteins is thus a prerequisite to an understanding of the physiological functioning of the tissue and to the changes that occur in it during health and disease.

It has been mentioned previously that methods for extracting protein–polysaccharide from cartilage that depend on stirring the tissue in salt solutions of low concentration or disrupting it mechanically by high-speed homogenization have certain disadvantages (Mason & Mayes, 1973*b*). Thus the observation originally made by Sajdera & Hascall (1969) and subsequently confirmed by other workers (e.g. Rosenberg *et al.*, 1970*a*; Mason & Mayes, 1973*b*) that up to 80% of the protein–polysaccharide of bovine nasal cartilage can be extracted by stirring or shaking the tissue in a salt solution of a critical high ionic strength, would seem to provide the best approach for obtaining protein–polysaccharide preparations from the tissue. Moreover, the use of equilibrium-density-gradient centrifugation methods for separating both unbound and ionically bound protein from the extracted protein–polysaccharide (Hascall & Sajdera, 1969) has enabled a major fraction (termed the proteoglycan subunit fraction) of these molecules to be isolated and used for further studies (see, e.g., Hascall & Sajdera, 1970; Rosenberg *et al.*, 1970*a*,*b*).

The analyses of three such proteoglycan fractions isolated from three different high-ionic-strength salt extractions of cartilage from the same batch of tissue show only small differences. The protein content, for example, increases from 5.1% for proteoglycan subunit extracted with $CaCl_2$ to 6.1% for $LaCl_3$ extracts and to 7.3% for guanidinium chloride extracts. Statistical analysis indicates that the difference in protein content between these extracts in $CaCl_2$ and $LaCl_3$ is significant only at probabilities $0.1 > P > 0.05$ (see Table 4 for S.D.). The probability level for the difference between the protein content of proteoglycan subunit extracted with $LaCl_3$ and that extracted with guanidinium chloride is of the same order. However, the difference between the protein content of the $CaCl_2$ and guanidinium chloride extracts is significant at the probability level $P < 0.001$. Thus although the difference in protein content of these two proteoglycans is small, it is unlikely to be due to variation in analytical technique alone.

Another small difference in the composition of proteoglycan subunit ($CaCl_2$ extract) and proteoglycan subunit (guanidinium chloride extract) is revealed in their glucosamine:galactosamine ratios (see Table 1). A smaller ratio was consistently found for the former ($CaCl_2$ extract) in proteoglycan fractions isolated from other batches of bovine nasal

cartilage as well as for the one reported above. The results suggest that the guanidinium chloride extract may be relatively enriched in the glycosaminoglycan, keratan sulphate, compared with the $CaCl_2$ extract (see Mason, 1970).

Hascall & Sajdera (1970) have demonstrated that the proteoglycan subunit fraction isolated from guanidinium chloride extracts of bovine nasal cartilage is heterogeneous in composition. They report that a minor component (3%, w/w) of the fraction is clearly enriched in its polypeptide and keratan sulphate content compared with the overall analysis of the proteoglycan subunit fraction. It seems possible therefore that the small differences between the protein content and hexosamine ratios of the $CaCl_2$ and guanidinium chloride extracts of proteoglycan subunit may be due to a slightly enhanced extraction of this component from the tissue by 4.0M-guanidinium chloride.

The use of prolonged centrifugation times in preparing proteoglycan subunit fractions from protein–polysaccharide isolated from the three different high-ionic-strength extracts was undertaken to eliminate the possibility that equilibrium conditions are not reached during the 48h centrifugation time described by Hascall & Sajdera (1969). Since the centrifugation is carried out in 4.0M-guanidinium chloride each protein–polysaccharide preparation would be present as its guanidinium salt, irrespective of its source. There appears to be little difference analytically (Tables 1, 2 and 3) between a fraction isolated after a 48h centrifugation and a 96h centrifugation. Thus for most practical purposes it may be assumed that equilibrium conditions for the preparation of the proteoglycan subunit fraction from protein–polysaccharide extracted with solutions of high ionic strength are attained in the shorter time-period.

The amino acid composition of the proteoglycan subunit fraction isolated by us from a 4.0M-guanidinium chloride extract shows very close agreement with that reported for a similar fraction by Hascall & Sajdera (1969), but less agreement with a later analysis reported by these authors (Hascall & Sajdera, 1970). Two differences are apparent, however, between our analysis and that of Hascall & Sajdera (1969). First, the histidine content of our preparations (Table 2) is lower than that reported by Hascall & Sajdera (1969). Particular care was taken in our amino acid analysis to ensure that a number of minor ninhydrin-positive compounds that emerge in the basic region of the chromatogram but are not identifiable with basic amino acids were completely resolved from the latter. Resolution of these compounds, which are presumed to be degradation products of hexosamine, may have enabled us to obtain a more accurate measurement of histidine.

Secondly, half-cystine residues in our preparations were always present in quantities of approx. 1–2mol/1000mol of amino acid compared with a value of 9mol/1000mol reported by Hascall & Sajdera (1969). This difference is particularly noteworthy, since Hascall & Sajdera (1969) have concluded from reduction and alkylation experiments that intact disulphide bonds in the proteoglycan subunit fraction are a prerequisite to its ability to form specific aggregates with a glycoprotein.

The amino acid analysis of the proteoglycan fractions from the high-ionic-strength inorganic salt extracts also contained only traces of half-cystine (Table 2). Moreover, another proteoglycan fraction, which has been isolated from bovine nasal cartilage by Rosenberg *et al.* (1970*a*), and designated PPL3 by them, contains only trace amounts of cystine. The discrepancy between our analyses and those of Hascall & Sajdera (1969) on the cystine content of the proteoglycan subunit fraction would appear therefore to merit further investigation.

It has been mentioned above that the proteoglycan subunit fraction isolated from 4.0M-guanidinium chloride extracts of bovine nasal cartilage exhibits compositional heterogeneity, fractions containing more keratan sulphate being associated with increased protein contents (Hascall & Sajdera, 1970). This heterogeneity was demonstrated by equilibrium-density-gradient centrifugation, the fraction most enriched in keratan sulphate having a density of less than 1.6g/ml. However, the protein–polysaccharide preparation from which the proteoglycan subunit fraction was isolated has a density greater than 1.72g/ml (Hascall & Sajdera, 1969). This infers that the keratan sulphate-enriched component isolated from the proteoglycan subunit fraction must initially be firmly bound to another component of the protein–polysaccharide preparation to sediment with the latter when it is separated from the cartilage extract in the first centrifugation step. It seems likely therefore that the keratan sulphate-enriched proteoglycan, as well as the other components of the proteoglycan subunit fraction, must undergo some form of complex dissociation phenomenon (see Mason & Mayes, 1973*b*) when it is extracted from the cartilage matrix by high-ionic-strength salt solutions.

Previous investigations have indicated that salt solutions of higher ionic strength are required to extract keratan sulphate-enriched proteoglycans from hyaline cartilage than are required to extract at least a proportion of the proteoglycans containing almost exclusively chondroitin sulphate (Tsiganos & Muir, 1969). Moreover, the proteoglycans containing keratan sulphate were of larger molecular size than the chondroitin sulphate proteoglycans extracted at low ionic strength, suggesting that the former may be predominantly associated with macromolecular complexes whereas at least some of the latter are not. Low-ionic-strength salt solutions would not be expected to dissociate such complexes but would

perhaps extract non-aggregated molecules. It was therefore decided in the present investigation to isolate the proteoglycan subunit fraction from the small proportion of protein–polysaccharide extracted from the tissue with 0.15M-KCl (see the Experimental section) and to compare its composition with the proteoglycan fraction isolated from high-ionic-strength extracts of the cartilage.

The results of this analysis (Tables 1, 2, 4 and 5) show that the composition of the proteoglycan subunit fraction isolated from low-ionic-strength extracts of the cartilage is different from that isolated from high-ionic-strength extracts. The protein content of the 0.15M-KCl-extracted proteoglycan is considerably lower than that of the proteoglycans extracted at high ionic strength (Table 4). Similarly its glucosamine content is much smaller, whereas its glucuronic acid and galactosamine content is increased (Table 1), indicating that it is relatively enriched in chondroitin sulphate and very low in keratan sulphate content. This is reflected in the high glucosamine:galactosamine ratio of the low-ionic-strength-extract proteoglycan (Table 1).

Prolonged centrifugation times of the protein–polysaccharide extracted by 0.15M-KCl appear to decrease the glucosamine:galactosamine ratio of the proteoglycan subunit that was subsequently isolated (Table 1). This may be due to trace quantities of a keratan sulphate-enriched component of the protein–polysaccharide taking a longer time to reach equilibrium-sedimentation conditions than the major chondroitin sulphate-enriched component found in the 48h preparation of the proteoglycan. This interpretation would be consistent with the glucosamine-enriched component having a lower density than that of the major component.

The amino acid analysis of the proteoglycan subunit fraction from low-ionic-strength extracts also shows features different from that of proteoglycans isolated from high-ionic-strength extracts. It is markedly enriched in serine and glycine but has a much lower content of aromatic and basic amino acids compared with the proteoglycan from high-ionic-strength extracts (Table 2). Half-cystine residues were not detected in the proteoglycan from low-ionic-strength extracts and it contains only traces of methionine. The difference between its amino acid composition and that of proteoglycans from high-ionic-strength extracts is also shown by the difference indices calculated for the different fractions (Table 5).

The proteoglycan subunit fraction isolated from the 0.15M-KCl extract of bovine nasal cartilage shows striking similarities to a low-molecular-weight (~300000) proteoglycan (termed fraction R) isolated from a 0.15M-potassium acetate extract of pig laryngeal cartilage by gel filtration (Tsiganos & Muir, 1969). Both have high glucosamine:galactosamine ratios and hexuronate contents, consistent with their being almost exclusively chondroitin sulphate proteoglycans. Their protein components both have high serine and glycine contents and contain no cystine. In view of this similarity, one interpretation of our results might be that the proteoglycan subunit fraction from low-ionic-strength extracts of bovine nasal cartilage may represent the fundamental non-aggregated chondroitin sulphate–proteoglycan of the tissue.

Simpson & Davidson (1972) treated a protein–polysaccharide preparation obtained from high-ionic-strength extracts of pig cartilage with collagenase. A proteoglycan fraction was isolated from the digest that had a high content of serine and glycine but a lower content of glucosamine than the original protein–polysaccharide preparation. Although some peptide cleavage had occurred in the proteoglycan fraction isolated, it seems possible that the fraction may represent a partial separation of an essentially chondroitin sulphate–proteoglycan from glucosamine-enriched components of protein–polysaccharide obtained from high-ionic-strength extracts of the cartilage.

The function of keratan sulphate-enriched proteoglycans remains obscure. Rosenberg *et al.* (1970*a*,*b*) have suggested that the considerable molecular-weight polydispersity of the proteoglycan subunit fraction isolated from high-ionic-strength extracts may be due to the presence of dimeric as well as monomeric forms of proteoglycan in the fraction. A speculative variation on this theme might be that the polydispersity is due to variable degrees of association between the chondroitin sulphate–proteoglycans which have been isolated from low-ionic-strength extracts of the tissue and the keratan sulphate-enriched proteoglycans which have been shown to be present in the proteoglycan subunit fraction isolated from high-ionic-strength extracts. Previous investigations have also suggested this possibility (see, for example, Tsiganos & Muir, 1969; Mason, 1971). Such associations would presumably be maintained by covalent bonds rather than by ionic bonds between the two proteoglycan species, although these would be unlikely to be of a disulphide nature (Hascall & Sajdera, 1970).

Moreover, since the available evidence suggests that cystine is absent in chondroitin sulphate–proteoglycans it must reside in the keratan sulphate-enriched component of the proteoglycan subunit fraction from high-ionic-strength extracts. A linkage between keratan sulphate–proteoglycans and chondroitin sulphate–proteoglycans would therefore include the cystine residues in the overall structure of the macromolecule, which are considered to be a requirement for the formation of aggregates between proteoglycans and other proteins (Hascall & Sajdera, 1969).

Finally, variable degrees of association between a

predominantly chondroitin sulphate–proteoglycan species and a keratan sulphate–proteoglycan species would be reasonably consistent with the observed compositional heterogeneity of protein–polysaccharide preparations (see, e.g., Mashburn & Hoffman, 1971).

We are grateful to the Nuffield Foundation for financial support.

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