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Cartilage Proteoglycans

STRUCTURE AND HETEROGENEITY OF THE PROTEIN CORE AND THE EFFECTS OF SPECIFIC PROTEIN MODIFICATIONS ON THE BINDING TO HYALURONATE

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Purified proteoglycans extracted from pig laryngeal cartilage in 0.15M-NaCl and 4M-guanidinium chloride were analysed and their amino acid compositions determined. Selective modification of amino acid residues on the protein core confirmed that binding to hyaluronate was a function of the protein core, and was dependent on disulphide bridges, intact arginine and tryptophan residues, and ε-amino groups of lysine. Fluorescence measurement suggested that tryptophan was not involved in direct subsite interactions with the hyaluronate. The polydispersity in size and heterogeneity in composition of the aggregating proteoglycan was compatible with a structure based on a protein core containing a globular hyaluronate-binding region and an extended region of variable length also containing a variable degree of substitution with chondroitin sulphate chains. The non-aggregated proteoglycan extracted preferentially in 0.15M-NaCl, which was unable to bind to hyaluronate, contained less cysteine and tryptophan than did other aggregating proteoglycans and may be deficient in the hyaluronate-binding region. Its small average size and low protein and keratan sulphate contents suggest that it may be a fragment of the chondroitin sulphate-bearing region of aggregating proteoglycan produced by proteolytic cleavage of newly synthesized molecules before their secretion from the cell.

Cartilage proteoglycans are complex macromolecules in which many chondroitin sulphate and keratan sulphate chains are attached at their reducing-terminal ends to a polypeptide backbone. Within each preparation the extent of substitution of the protein core with glycosaminoglycan chains varies, producing a polydisperse population of molecules with differences in molecular weight, composition and physical properties (Hascall & Sajdera, 1970; Tsiganos *et al.*, 1971). There are also small but significant differences in amino acid composition between different proteoglycan fractions, suggesting some heterogeneity of the protein core (Tsiganos *et al.*, 1971). Cartilage proteoglycans have been shown to form aggregates (Hascall & Sajdera, 1969) in which many proteoglycans bind to a chain of hyaluronic acid (Hardingham & Muir, 1972*a*, 1973, 1974; Hascall & Heinegård, 1974*a*,*b*). Two other protein components, known as 'protein links', also bind to the aggregate and appear to increase its stability in the ultracentrifuge (Gregory, 1973; Hardingham & Muir, 1972*a*) and when examined by other methods (Hardingham & Muir, 1975).

The binding to hyaluronic acid has been shown to involve a single specific site on the proteoglycan protein core (Hardingham & Muir, 1972*a*), which had a high affinity for a decasaccharide unit of hyaluronate (Hardingham & Muir, 1973; Hascall & Heinegård, 1974*b*). The heterogeneity of protein cores previously reported in disaggregated proteoglycans has been investigated here in more detail, and the nature of the hyaluronate-binding site examined by selective chemical modification of amino acid residues.

Experimental

Materials

All reagents were of analytical grade, except for glucosamine hydrochloride, galactosamine hydrochloride, glucuronolactone, carbazole, guanidinium chloride and *N*-bromosuccinimide which were of reagent grade. The guanidinium chloride was purified with activated charcoal (Norit N.K.; Hopkin and Williams, Chadwell Heath, Essex, U.K.), and *N*-bromosuccinimide was recrystallized from acetic acid (Spande & Witkop, 1967). Hyaluronate (sodium salt) was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. The reagents for protein modification, namely 5,5′-dithiobis-(2-nitrobenzoic acid), 2-nitrophenylsulphenyl chloride, 2,4,6-trinitrobenzenesulphonic acid, *p*-chloromercuribenzoic acid, iodoacetamide and butane-2,3-dione (diacetyl) were obtained from Sigma (London) Chemical Co., Kingston-on-Thames, Surrey, U.K. 2-Methylmaleic anhydride (citraconic anhydride) was obtained from

Koch–Light Laboratories, Colnbrook, Bucks., U.K. Dithiothreitol and amino acid standards were obtained from Calbiochem Co., Hereford, U.K.

General analytical procedures

Uronic acid was determined by an automated procedure (Heinegård, 1973) of the modified (Bitter & Muir, 1962) carbazole reaction (Dische, 1947), by using glucuronolactone as standard. Protein was measured by an automated modification (Heinegård, 1973) of the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V) as standard, and by the summation of individual amino acids. Protein values estimated by the colorimetric method were 40–50% higher than those by the latter method.

Glucosamine/galactosamine molar ratios were determined after hydrolysis in 8M-HCl for 3h at 95°C (Swann & Balazs, 1966) by automated ion-exchange chromatography as previously described (Hardingham & Muir, 1974).

Amino acid analysis

Total amino acid analyses were performed on a Locarte amino acid analyser by using a single-column elution system, after hydrolysis in 6M-HCl for 24h at 105°C, in sealed glass tubes under N_2, as previously described (Hardingham & Muir, 1974). No corrections were made for the destruction of amino acids during hydrolysis. Hydroxyproline was determined by the method of Woessner (1961) after hydrolysis in 6M-HCl at 105°C for 24h in sealed glass tubes under N_2. Cystine was measured after oxidation with performic acid to cysteic acid (Hirs, 1967), followed by hydrolysis in 6M-HCl for 24h at 105°C in glass tubes sealed under N_2. Cysteic acid was determined automatically by using the column (15cm×1cm) of a Locarte amino acid analyser packed with Dowex 1 (X8; minus 400 mesh; Cl^- form) anion-exchange resin eluted with 0.01M-HCl (60ml) followed by 0.1M-HCl (90ml). Amounts as low as 5nmol could be determined.

Methionine was measured as the sulphone, after oxidation (Hirs, 1967) and subsequent acid hydrolysis, by using the three-buffer single-column elution system, as for conventional amino acid analysis. The methionine sulphone was eluted between aspartic acid and threonine.

Tryptophan was determined after alkaline hydrolysis in 4.2M-NaOH for 16h at 110°C, and subsequent neutralization by the ion-exchange method of Hugli & Moore (1972).

Sequential extraction of proteoglycan with 0.15M-NaCl followed by 4M-guanidinium chloride

Proteoglycans were extracted sequentially from pig laryngeal cartilage, essentially as described previously (Hardingham & Muir, 1974). Fresh pig laryngeal cartilage sliced with a Stanley Surform at 4°C was suspended in ten times its weight of cold 0.15M-NaCl, pH6.8, and agitated for 3h at 4°C. The extract was filtered and the residue washed twice with 30ml of cold 0.15M-NaCl, pH6.8. Extract and washings were combined and concentrated by Diaflo ultrafiltration through a UM-10 membrane. The proteoglycans were then purified by density-gradient centrifugation as described below. The residual cartilage after extraction with 0.15M-NaCl, pH6.8, was suspended in ten times its weight of 4M-guanidinium chloride 0.013M-citric acid/0.012M-Na_2HPO_4 buffer, pH4.5, and extracted for 24h at 4°C. The extract was filtered and the residual cartilage washed twice with small volumes of citrate/phosphate-buffered 4M-guanidinium chloride. The extract (plus washings) was adjusted to pH5.8 and dialysed against 7 vol. of 0.05M-sodium acetate, pH5.8. The proteoglycans were then purified by density-gradient centrifugation as described below.

Dissociative density-gradient centrifugation of 0.15M-NaCl extract

To the concentrated 0.15M-NaCl extract was added enough 8.0M-guanidinium chloride solution in 0.05M-sodium acetate, pH5.8, so that the final guanidinium chloride concentration was 4.0M, and the density was adjusted to 1.508g/ml by the addition of solid CsCl. The proteoglycan concentration was approx. 3mg/ml. Equilibrium density-gradient centrifugation was performed in an MSE 65 centrifuge with an 8×25ml titanium angle rotor at 95000$g_{av.}$ for 48h at 20°C. The tubes were then quickly frozen in a solid-CO_2/acetone bath and cut into three fractions, bottom (ND1), middle (ND2) and top (ND3), containing 4ml, 10ml and 4ml respectively. These fractions were dialysed against 50mM-sodium acetate, pH6.8, and stored at −20°C for further investigation.

Associative equilibrium density-gradient centrifugation of proteoglycans extracted with 4M-guanidinium chloride

To the dialysed 4M-guanidinium chloride extract, containing 3–4mg of proteoglycan/ml, was added solid CsCl until the density of the solution was 1.60g/ml. Density-gradient centrifugation was then performed as described above. After centrifugation the tubes were quickly frozen in a solid-CO_2/acetone bath and cut into two fractions, a bottom fraction (A1; 6ml) and a top fraction (A2; 12ml). Samples of fractions A1 and A2 were dialysed against 0.05M-sodium acetate, pH5.8, and were stored frozen for further investigation. The non-diffusible material in the top fraction A2 was concentrated by Diaflo

ultrafiltration through a UM 10 membrane to a final concentration of 3–4mg of proteoglycan/ml, and the density adjusted to 1.50g/ml by the addition of solid CsCl. After centrifuging for a further 48h, exactly as described above, the tubes were frozen in a solid-CO_2/acetone bath and again cut into two fractions, A2-1 and A2-2. The combined bottom fractions (A1 and A2-1) were dialysed against 0.05M-sodium acetate, pH5.8, and were stored frozen for further investigation.

Fractionation of proteoglycans by equilibrium density-gradient centrifugation under dissociative conditions

A solution (3–4mg/ml) of proteoglycan fraction (A1+A2-1), containing mainly aggregated proteoglycans, was mixed with an equal volume of 8M-guanidinium chloride, and solid CsCl was then added to a density of 1.50g/ml. The solution was then centrifuged under dissociative conditions as described above for the 0.15M-NaCl extract. After centrifugation the tubes were frozen and cut into three fractions, bottom 4ml (D1), middle 10ml (D2) and top 4ml (D3). Samples of each fraction were dialysed against 0.05M-sodium acetate, pH5.8. Guanidinium chloride solution (4M) was added to the bulk of fraction D1 until the final concentration of guanidinium chloride was 2M. Solid CsCl was then added until the density of the solution was 1.80g/ml. This solution was then centrifuged for 48h as described above. The contents of each tube were removed by piercing the tube and pumping upwards an inert fluorocarbon liquid (Flutec PP9, ISC Chemicals Ltd., Avonmouth, Bristol, U.K.) and collecting 3ml fractions. The density of the fraction at the top of the tube was 1.691g/ml, and that of the bottom fraction 1.833g/ml. Fractions of similar density from each tube were pooled, giving six discrete fractions D1-1 to D1-6 from the bottom to the top of the tubes respectively. Each fraction was dialysed against 0.05M-sodium acetate, pH6.8, and kept frozen for further investigation.

Optical methods

The absorption spectra of proteoglycan samples were measured against appropriate buffer blanks in quartz cells of 1cm light-path, in a Beckman DB-G or Cecil 272 spectrophotometer. Fluorescence of samples was measured by using an Aminco–Bowman spectrofluorimeter.

Gel chromatography

Samples of proteoglycan (0.5ml–1.5ml), containing 1.0–1.5mg of uronic acid in 0.5M-sodium acetate, pH6.8, were applied to a column (165cm × 1.1cm) of Sepharose 2B (Pharmacia, Uppsala, Sweden), which was eluted upwards with 0.5M-sodium acetate, pH6.8, at 4°C at 6ml/h by using a peristaltic pump. Fractions (40 drops; about 2.5ml) were collected, and their uronic acid and/or protein content was determined. Proteoglycan aggregates and glucuronolactone were used as markers of the void volume and total column volume respectively.

Binding of proteoglycan fractions to hyaluronate

Samples of proteoglycan containing 0.8–1.2mg of uronic acid in 0.8–1.5ml of non-dissociative buffer, pH6.0–7.0, either (*a*) 0.5M-guanidinium chloride containing 0.05M-sodium acetate, (*b*) 0.1M-sodium phosphate, or (*c*) 0.5M-sodium acetate, were mixed with hyaluronate containing 8–12μg of uronic acid (i.e. 1% of the uronic acid in the proteoglycan). After mixing at 20°C for 15min, samples were frozen and stored, or chromatographed at once on a column of Sepharose 2B at 4°C as described above. The proportion of proteoglycan bound to hyaluronate was determined from the amount of uronic acid eluted in the region of the void volume of the column compared with the control proteoglycan chromatographed in the absence of hyaluronate (Hardingham & Muir, 1972*a*, 1974).

Viscometry

Measurement of the viscosity of proteoglycan solutions was made with an Ostwald capillary viscometer in a viscometer bath (Townson and Mercer, Croydon, Surrey, U.K.) at 30°C as previously described (Hardingham & Muir, 1972*a*).

Chemical modification of the proteoglycan protein core

All modifications were carried out on disaggregated proteoglycan fraction (D1) prepared as described above. The concentration of proteoglycan solutions was determined from their hexuronate content, which accounted for 24.8% of the dry weight (Hardingham & Muir, 1974). The molarity of solutions was calculated from the number-average molecular weight (M_n) of the proteoglycan. This was calculated to be approx. 1.63×10^6 from the results of Hascall & Sajdera (1970), in which it was shown that proteoglycans from bovine nasal septa had a normal or Gaussian molecular-weight distribution, with a mean of $2.54\times10^6\pm1.16\times10^6$ (S.D.). Comparison of the behaviour in the analytical ultracentrifuge showed that proteoglycans from pig laryngeal cartilage had properties similar to those from bovine nasal septa ($s_0 = 24.1$S for the former, 25.0S for the latter; T. E. Hardingham & H. Muir, unpublished work). The results of Hascall & Sajdera (1970) also showed the mol.wt. of the protein core to be approx. 200000;

this was used as an average value in calculating the number of amino acid residues per mol of the proteoglycan in fraction D1.

(1) *Reduction and alkylation of disulphide bridges.* A sample of proteoglycan in 4M-guanidinium chloride/0.05M-Tris/HCl, pH9.0, was incubated at 37°C for 3h in the presence of 10mM-dithiothreitol and then alkylated with 40mM-iodoacetamide for 16h at 20°C in the dark (Hascall & Sajdera, 1969). The sample was then dialysed against 0.05M-guanidinium chloride/0.05M-sodium acetate, pH5.8, and its limiting viscosity number determined in an Ostwald capillary viscometer.

(2) *Reduction and re-oxidation of disulphide bridges.* Proteoglycan was mixed with hyaluronate (156:1, w/w) and incubated at 30°C in 0.5M-guanidinium chloride/10mM-dithiothreitol in 0.05M-Tris/HCl, pH9.0, or 0.05M-sodium acetate, pH5.8. The fall in viscosity was monitored in an Ostwald capillary viscometer and when it had ceased, the sample was removed from the viscometer and the dithiothreitol was removed by ultrafiltration in a Diaflo cell by using several volumes of 0.05M-guanidinium chloride. To re-oxidize the disulphide bridges the cell was pressurized ($\equiv 3.5\times10^5$Pa) with O_2 instead of N_2. The proteoglycan/hyaluronate solution was diluted to its former concentration with 0.5M-guanidinium chloride/0.05M-sodium actate, pH5.8, and its viscosity re-determined.

A similar reduction/re-oxidation cycle was also carried out on proteoglycan in the absence of hyaluronate. The ability of samples of it to interact with hyaluronate was tested in a viscometer before and after treatment.

(3) *Reaction with p-chloromercuribenzoate.* Proteoglycan was treated with *p*-chloromercuribenzoate as described by Riordan & Vallee (1967). To 2ml of the proteoglycan solution (10mg/ml) in 0.1M-sodium phosphate buffer, pH7.0, was added 3ml of 0.3mM-*p*-chloromercuribenzoate. The extinction of the proteoglycan solution was monitored at 250nm for 2h. A similar reaction with *p*-chloromercuribenzoate was also carried out on proteoglycan in 8M-urea, pH7.0. After dialysis against 0.1M-sodium phosphate, pH7.0, samples of the treated proteoglycans and controls were mixed with hyaluronate and the interaction was examined by gel chromatography on Sepharose 2B.

(4) *Reaction with 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959).* To a sample of 3ml of proteoglycan (fraction D1) in 0.1M-sodium phosphate buffer, pH7.0, was added 20μl of 10mM-5,5′-dithiobis-(2-nitrobenzoic acid) at 20°C. The increase in absorbance was followed at 412nm, and the extent of reaction calculated assuming $\varepsilon_{412} = 13600$ litre$\cdot$mol$^{-1}\cdot$cm^{-1} (Ellman, 1959). After 3h a sample was dialysed against 0.1M-sodium phosphate, pH7.0, mixed with hyaluronate, and examined by gel chromatography on Sepharose 2B. Reaction was also carried out in 0.3% (w/v) sodium dodecyl sulphate/0.05M-sodium acetate, pH5.8 (Kemp & Forest, 1968).

(5) *Acetylation of proteoglycan with acetic anhydride.* The method used was that of Riordan & Vallee (1967). Samples of proteoglycan in either half-saturated sodium acetate adjusted to pH8.5 with 0.1M-NaOH, or 0.1M-sodium borate buffer, pH8.5, were cooled in an ice bath and 20μl portions of acetic anhydride were added with mixing. The solution was maintained between pH7.5 and pH8.5 by the addition of 1.0M-NaOH. The total amount of acetic anhydride added was up to 1000mol/mol of proteoglycan (i.e. about 40 times the concentration of lysine residues) and it was added over 30min. After 1–2h the samples were dialysed against 0.1M-sodium phosphate buffer, pH7.0, and a sample was mixed with hyaluronate and examined by gel chromatography on Sepharose 2B.

(6) *Reaction of proteoglycan with 2-methylmaleic (citraconic) anhydride.* Reaction was carried out under the conditions described by Dixon & Perham (1968). To 2ml of proteoglycan solution (10mg/ml) in 0.1M-sodium phosphate buffer, pH8, in an ice bath was added up to 200μl of 2-methylmaleic anhydride in 20μl batches over 30min. The solution was maintained between pH7.5 and pH8.0 by the addition of 1.0M-NaOH. After 60min the sample was divided in two. Half was dialysed against 0.1M-sodium phosphate, pH7.0, and the remainder was adjusted to pH3.0 with 1M-HCl and left overnight at 20°C to remove the 2-methylmaleyl groups (Dixon & Perham, 1968). The solution was then re-adjusted to pH7.0, and samples of the modified proteoglycan before and after acid treatment were mixed with hyaluronate and examined by gel chromatography on Sepharose 2B.

(7) *Reaction of proteoglycans with butane-2,3-dione (diacetyl).* Proteoglycan was treated with butane-2,3-dione in borate buffer (Yankeelov, 1970; Riordan, 1973). (*a*) To the proteoglycan (10mg/ml) in 0.5M-sodium borate buffer, pH8.5, was added butane-2,3-dione at various concentrations up to 100mM. After 2.5h at 20°C the solution was dialysed against 0.5M-borate buffer, pH8.5. (*b*) Proteoglycan at similar concentration was also treated with 100mM-butane-2,3-dione in 0.5M-sodium phosphate, pH6.0, in the dark for 6h at 20°C or (*c*) with 100mM-butane-2,3-dione in 0.5M-sodium borate, pH8.5, at 4°C for 15min. After the treatments the proteoglycan was dialysed against 0.1M-sodium phosphate, pH7. The extent of modification of arginine residues was determined by the loss of arginine on amino acid analysis under standard conditions and with the addition of phenol (100 times the tyrosine content) before hydrolysis (Yankeelov, 1970). Samples of all modified proteoglycans were mixed with hyaluronate and examined by gel chromatography on Sepharose 2B.

(8) *Reaction of proteoglycan with N-bromosuccinimide.* Proteoglycans were treated with *N*-bromosuccinimide under the conditions described by Spande & Witkop (1967). The proteoglycan (10mg/ml ≡ 6.1nM) in 0.1M-sodium acetate buffer, pH4.0, was treated with *N*-bromosuccinimide (5.6mM) in water, added in 5μl portions. The reaction was followed by the change in the u.v. spectrum between 240 and 320nm. *N*-Bromosuccinimide was added until there was no longer any decrease in absorbance at 280nm. The tryptophan content was calculated as described by Spande & Witkop (1967). Similar samples of proteoglycan were then treated with 10%, 50%, 100% and 200% of the amount of *N*-bromosuccinimide required to give the maximum ΔE_{280}. After dialysis against 0.1M-sodium phosphate buffer, pH6.8, the samples were mixed with hyaluronate and examined by gel chromatography on Sepharose 2B.

(9) *Reaction of proteoglycan with 2-nitrophenylsulphenyl chloride.* Proteoglycans were treated with 2-nitrophenylsulphenyl chloride by a modification of the method described by Scoffone *et al.* (1968). To 3 ml of a solution of proteoglycan (6.7mg/ml) in 33% (v/v) acetic acid was added 200μl of 2-nitrophenylsulphenyl chloride (3mg/ml in acetic acid). After 16h at 20°C the solution was dialysed against 0.1M-sodium phosphate buffer, pH7.0. The spectrum of the modified proteoglycan in 80% acetic acid and the E_{365} were recorded. The extent of reaction was calculated by assuming $\varepsilon_{365} = 4000$ litre·mol^{-1}·cm^{-1} (Scoffone *et al.*, 1968). A sample of proteoglycan was also treated overnight with 37% (v/v) acetic acid as a control. Control and modified samples were mixed with hyaluronate and examined by gel chromatography on Sepharose 2B.

(10) *Effect of heat on the hyaluronate-binding site of the proteoglycan.* Proteoglycan was heated at 80°C or 100°C in 0.5M-guanidinium chloride/0.05M-sodium acetate, pH5.8. Samples were removed at various times, cooled to 20°C and mixed with sufficient hyaluronate to bind all the proteoglycan. The decrease in the proportion of proteoglycan able to bind was determined by gel chromatography on Sepharose 2B as described above. From a semi-log plot of the results, the half-life of the binding site was determined. Similar measurements were also made on proteoglycan after heating at 80°C in 0.15M-NaCl/0.01M-sodium phosphate, pH7.2.

Determination of total free amino groups

Total free amino groups in samples of some control and modified proteoglycan were determined by reaction with 2,4,6-trinitrobenzenesulphonic acid as described by Habeeb (1967). E_{335} of the samples was recorded, and the total amino groups were determined by using L-leucine as standard.

Results

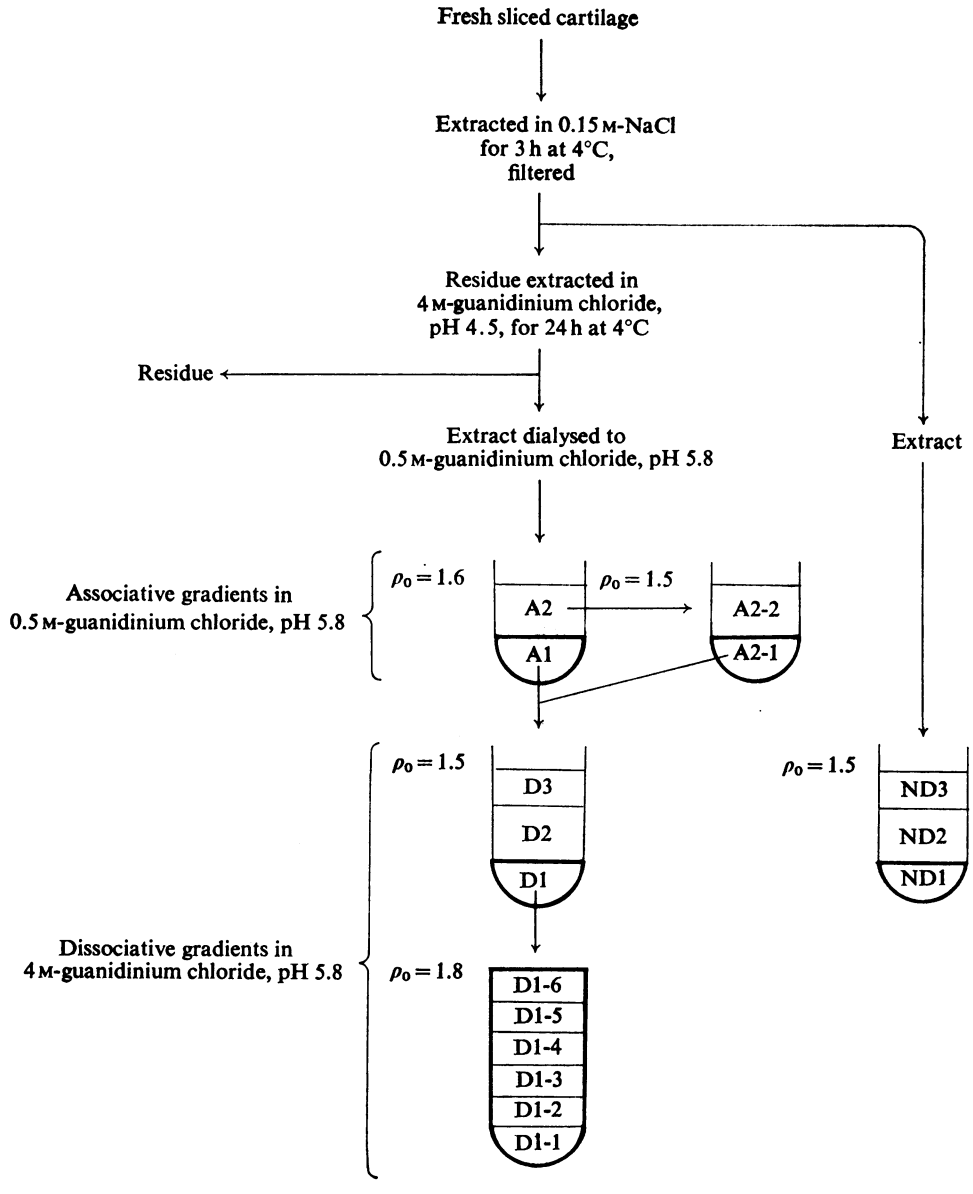
Extraction, purification and fractionation of proteoglycans

Proteoglycans were extracted sequentially from cartilage as described by Hardingham & Muir (1974) (Scheme 1). Extraction with 0.15M-NaCl at 4°C for 3h was shown to extract preferentially non-aggregated proteoglycans that were unable to bind to hyaluronic acid and were of smaller average hydrodynamic size than the remainder. To remove all non-covalently bound protein, proteoglycans were purified by equilibrium density-gradient centrifugation under dissociative conditions [density (ρ) = 1.50g/ml], when 94.4% of the uronic acid was recovered in the purified fraction ND1 (Table 1).

The cartilage residue after extraction with 0.15M-NaCl was then extracted with 4M-guanidinium chloride, buffered at pH4.5, which dissociates aggregates and extracts a large proportion of the proteoglycan in the tissue (Hascall & Sajdera, 1969). Aggregates were then allowed to re-form during dialysis, and were purified by equilibrium density-gradient centrifugation under associative conditions with a starting density of 1.60g/ml. After 48h centrifugation 86.8% of the hexuronic acid was in the bottom third of the gradient (fraction A1) (Table 2), but to account for as much as possible of the proteoglycans in subsequent analyses, the top fraction (A2) was centrifuged again in 0.5M-guanidinium chloride with a lower starting density of 1.50g/ml, when 64% of the remaining uronic acid separated in the bottom third of the gradient (fraction A2-1) (Table 2). The combined fractions A1 and A2-1 thus accounted for 95.3% of the total hexuronic acid that was extracted with 4M-guanidinium chloride. The aggregates in this combined fraction were then dissociated, and the protein-link component and hyaluronate were separated from the proteoglycan by density-gradient centrifugation in 4M-guanidinium chloride (Hardingham & Muir, 1974) (Table 3). The proteoglycan fraction (D1) was further fractionated under dissociative conditions in a gradient of higher starting density (1.80g/ml) into six fractions (D1-1 to D1-6) which varied in density from 1.833 to 1.691g/ml (Table 3).

Analysis of proteoglycan fractions

Protein and uronic acid contents and galactosamine/glucosamine molar ratios of the various disaggregated proteoglycan fractions were determined (Table 3). Since the lengths of chondroitin sulphate chains were found to be rather similar in fractions of different buoyant density (Tsiganos *et al.*, 1971), the very large variation in hexuronic acid/protein ratio represents large differences in the degree of substitution of the polypeptide backbone with chon-



Scheme 1. *Preparation and fractionation of proteoglycans from pig laryngeal cartilage*

droitin sulphate chains. The proteoglycans in fraction D1-1 therefore contain 3.5 times as many chondroitin sulphate chains per unit weight of protein as do the proteoglycans in fraction D1-6. The lower chondroitin sulphate content of the protein-rich fractions was reflected in the glucosamine/galactosamine molar ratios, but since the ratio of glucosamine to protein was fairly constant among different fractions, the extent of substitution of the protein core by keratan sulphate varied little.

Gel chromatography on Sepharose 2B showed that all disaggregated fractions were included by this gel and were polydisperse (Fig. 1*a*). As interaction with hyaluronate produces fractions excluded from Sepharose 2B (Hardingham & Muir, 1972*a*, 1974), it would appear that all endogenous hyaluronate had

Table 1. *Analysis of proteoglycans extracted with* 0.15 M-*NaCl and fractionated by equilibrium density-gradient centrifugation under dissociative conditions*

Fractions are as shown in Scheme 1.

Fraction	Vol. (ml)	Content (% of total)		Hexuronic acid protein (weight ratio)	Galactosamine glucosamine (molar ratio)
		Hexuronic acid	Protein		
Extract	—	100	100	0.67	13.4
ND1	4	94.4	39.7	2.63	19.5
ND2	10	4.2	13.6	0.35	3.2
ND3	4	1.5	46.7	0.04	1.0

Table 2. *Analysis of proteoglycans extracted with* 4 M-*guanidinium chloride and fractionated by equilibrium density-gradient centrifugation under associative conditions*

Fractions are as shown in Scheme 1. Protein content is obtained from the summation of amino acid analyses.

Fraction	Vol. (ml)	Hexuronic acid (% of total)	Hexuronic acid protein (weight ratio)	Galactosamine glucosamine (molar ratio)
Extract	—	100	0.77	8.2
A1	6	86.8	2.08	9.6
A2	12	13.2	0.24	4.2
A2-1	6	8.5	0.43	—
A2-2	12	4.7	0.08	—
A1+A2-1	—	95.3	1.67	8.8

been removed from disaggregated proteoglycans. Among the chondroitin sulphate-rich fractions D1-1 to D1-4, as the protein content increased, their positions of elution ($K_{av.}$) increased, which indicates that hydrodynamic size decreased with decrease in chondroitin sulphate content. However, the non-aggregated fraction ND1 (extracted with 0.15 M-NaCl; see Scheme 1), although of even higher chondroitin sulphate content than fraction D1-1, was of smaller average size on gel chromatography than fraction D1-4, and thus did not fit in with the correlation of composition and size shown by the fraction series D1-1 to D1-6 (Fig. 1*a*). This difference in structure was further demonstrated by gel chromatography, which showed that, after the addition of excess of hyaluronate to each fraction, 72–77% of fractions D1-1 to D1-4 were able to bind to hyaluronate, whereas only 20.4% of fraction ND1 could do so (Table 4; Fig. 1b). As previously observed (Hardingham & Muir, 1974) the 0.15 M-NaCl extract contained a large proportion of non-aggregating proteoglycans.

The total amino acid composition of the different fractions is shown in Table 5. The fractions are arranged in order of decreasing chondroitin sulphate content. The majority of amino acids were present in similar amounts in all fractions. However, some changed continuously across the series, in particular the contents of serine, glycine, proline, arginine and phenylalanine. As the extensive density-gradient-preparation procedure should have ensured the removal of all non-covalently bound protein, and none was evident on gel chromatography (Fig. 1*a*), the progressive changes in amino acid composition across the series implied that the proteoglycans in different fractions did not have identical polypeptide cores.

Experiments of Heinegård & Hascall (1974*a*) with proteoglycans from bovine nasal cartilage suggest that the hyaluronate-binding region and the part bearing chondroitin sulphate chains form two distinct regions of the protein core, the former accounting for about two-fifths, and the latter for three-fifths of the protein, and it was proposed that the latter was of variable length (Heinegård & Hascall, 1974*b*). Analyses of chondroitin sulphate peptides obtained by exhaustive digestion with trypsin and chymotrypsin of cartilage proteoglycans from several sources show that serine, glutamate and glycine are the predominant amino acids around the chondroitin sulphate-protein linkage (Matthews, 1971). As shown in Table 5, the contents of these amino acids and to a lesser extent of proline were greater in proteoglycans with high chondroitin sulphate contents. On the other hand the contents of aspartate, alanine, valine, threonine, arginine and phenylalanine were higher in proteoglycans that contained a high proportion of protein. These amino acids may therefore be more predominant in the hyaluronate-binding regions. The analyses of fraction D1-1 to D1-6 are thus consistent with the model proposed by Heinegård & Hascall (1974*b*), but the characteristics of the proteoglycan (fraction ND1) extracted with 0.15 M-NaCl did not fit easily into this series. Although this proteoglycan contained more chondroitin sulphate and less protein than any other, it was unexpectedly of comparatively small average molecular size on gel chromatography ($K_{av.} = 0.40$), whereas it would have been of the largest size had it fitted the series exemplified by fractions D1-1 to D1-6. This suggests that the core protein of fraction

Table 3. *Analysis of proteoglycans extracted with 4M-guanidinium chloride and fractionated by equilibrium density-gradient centrifugation under dissociative conditions*

Fractions are as shown in Scheme 1. Protein content is from the summation of amino acid analyses.

Fraction	Vol. per tube (ml)	Content (% of total)		Hexuronic acid / protein (weight ratio)	Galactosamine / glucosamine (molar ratio)
		Hexuronic acid	Protein		
First gradient					
D1	4	94.4	55.8	2.5	12.3
D2	10	4.3	12.9	0.31	2.7
D3	4	1.3	31.3	0.06	1.6
Second gradient					
D1-1	3	32.2	24.1	3.13	16.4
D1-2	3	28.5	24.8	2.72	13.8
D1-3	3	17.2	14.9	2.57	12.5
D1-4	3	9.1	12.2	2.13	10.5
D1-5	3	8.7	13.5	1.30	8.7
D1-6	3	4.2	10.2	0.93	5.9

Table 4. *Gel chromatography on Sepharose 2B of proteoglycan fractions*

Gel chromatography was carried out as described in the text. Fractions are as shown in Scheme 1. There was insufficient of fraction D1-6 for gel chromatography.

Fraction	Distribution of hexuronic acid (% of total)			
	Without hyaluronate added		With hyaluronate added	
	Excluded	Retarded	Excluded	Retarded
A1	72.8	27.2	—	—
ND1	2	98	20.4	79.6
D1-1	0	100	76.6	23.4
D1-2	0	100	76.2	23.8
D1-3	0	100	75.7	24.3
D1-4	0	100	72.8	27.2
D1-5	0	100	—	—

ND1 is intrinsically different from the core proteins of fractions D1-1 to D1-6, and judging from the hydrodynamic size of the intact proteoglycan and its low protein content, was of much lower molecular weight.

The u.v. spectrum of the disaggregated proteoglycan fraction (see Fig. 3*a*) showed that tryptophan was present, and analysis after alkaline hydrolysis showed that all fractions contained 6–10 residues/1000 amino acid residues. Together with tyrosine, which accounted for about 18 residues/1000 amino acid residues, they accounted for 81% of the E_{280} of the proteoglycan.

Initial determination of the acid-labile amino acids, namely tryptophan, methionine and cysteine, showed that they were present in all fractions in low amounts, and there was little variation in their contents. As subsequent results showed that cystine bridges were an essential structural feature of the hyaluronate-binding region of the proteoglycan, and were not present in the chondroitin sulphate-bearing region, the presence of cysteine in the proteoglycan extracted with 0.15M-NaCl was examined in more detail. A sample of proteoglycan (fraction ND1) was fractionated by gel chromatography on Sepharose 2B in the presence of hyaluronic acid (Fig. 1*b*), which removed the small amount of proteoglycan that could bind to hyaluronate (at the void volume of the column), fraction ND1-A, from the major part which was unable to interact (the retarded material). The retarded material was divided into two fractions, with $K_{av.}$ greater than (fraction ND1-B), or less than (fraction ND1-C), 0.43. Analysis showed that the protein content of fractions ND1-B and ND1-C was much lower than of fraction ND1-A and the molar ratio of galactosamine to glucosamine was much higher in fractions ND1-B and ND1-C than in the starting material, fraction ND1 (Table 6). Fractions ND1-B and ND1-C were thus of lower protein and keratan sulphate content than the aggregating proteoglycans present in small amounts in fraction ND1. They also had a higher serine content, 164 residues/1000 amino acid residues, and much less cysteine, only about 3 residues/1000 amino acid residues. Their u.v. spectrum and the results of oxidation with *N*-bromosuccinimide suggested that, compared with other fractions, they contained less than four residues of tryptophan per 1000 amino acid residues.

Effect of chemical modification of the proteoglycan protein core on its ability to bind to hyaluronate

There is evidence that the hyaluronate-binding region of the core protein is of globular structure with disulphide bridges (Hascall & Heinegård, 1974*a*).

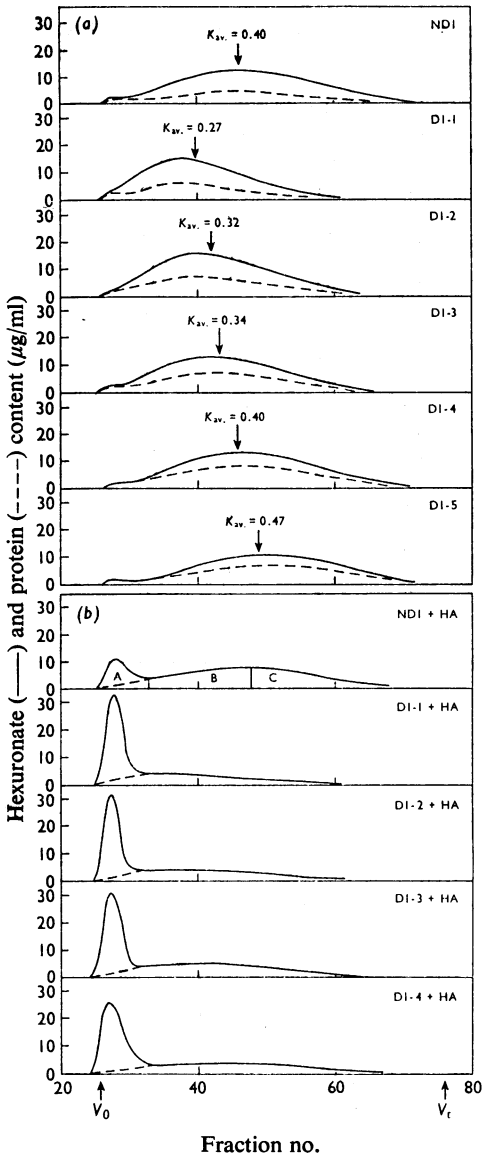


Fig. 1. *Gel chromatography on Sepharose 2B of subfractions of disaggregated proteoglycans (a) alone and (b) in the presence of 1% (w/w) hyaluronate*

Samples containing 1.0–1.5 mg of uronic acid were applied to a column (165 cm × 1.1 cm) of Sepharose 2B eluted as described in the text. The uronic acid (——) and protein (----) contents of the fractions (2.5 ml) were determined. V_0 and V_t mark the void volume and total volume of the column respectively. Fractions from ND1 in the presence of hyaluronate were pooled into three fractions A, B, C as shown for subsequent analyses (see Table 6).

The tertiary structure of this region is likely to be maintained by interactions among the constituent amino acid residues, and binding to hyaluronate will also involve specific subsite interactions between various amino acid residues of the protein core and groups on the hyaluronate chain. Hence various chemical modifications of amino acids were investigated that might prevent binding by altering the tertiary conformation of the binding site or by modifying specific groups involved in the subsite interactions with hyaluronate.

Cleavage of cysteine disulphide bridges. When the proteoglycans were reduced with dithiothreitol and alkylated with iodoacetamide, at least 80% of the cysteine residues were converted into carboxymethylcysteine, whereas none was formed by alkylation alone. Gel chromatography on Sepharose 2B of the reduced and alkylated proteoglycans showed a distribution of molecular size similar to that of the unmodified proteoglycans. As the hydrodynamic size and protein content of the proteoglycan were unaltered, the disulphide bridges appeared to be intramolecular rather than intermolecular and not in the major chondroitin sulphate-bearing region of the molecule. Similarly, determination of the limiting viscosity number of the reduced and alkylated proteoglycan also suggested that there was little change in structure (Fig. 2). The small decrease in limiting viscosity number may have reflected the presence of a trace of hyaluronate in the preparation of disaggregated proteoglycan used for the experiment.

When it had been reduced and alkylated, the proteoglycan could no longer interact with hyaluronate when mixed with it in optimum proportions, whereas after alkylation alone the proteoglycan was still able to bind. Thus the activity of the binding site depended on one or more cystine bridges.

The failure to detect carboxymethylcysteine in the amino acid analysis of the proteoglycan alkylated without reduction and the lack of reaction with *p*-chloromercuribenzoate or 5,5′-dithiobis-(2-nitrobenzoic acid) suggested that there were no free thiol groups in the native structure. The total content of disulphide bridges was thus about five per molecule of proteoglycan. However, the possibility of about one thiol group per molecule was suggested by reaction of 5,5′-dithiobis-(2-nitrobenzoic acid) in 0.3% sodium dodecyl sulphate (Kemp & Forest, 1968).

The rate of reduction of the disulphide bridge(s) necessary for the interaction with hyaluronate was examined in a viscometer at 30°C under mild conditions with 10 mM-dithiothreitol in 0.5 M-guanidinium chloride at pH 9.0 and also at pH 5.8. The loss of binding on reduction was complete in 90 min at pH 9 ($t_{\frac{1}{2}}$ = 14 min) and was more rapid than at pH 5.8 ($t_{\frac{1}{2}}$ = 102 min).

Table 5. *Amino acid composition of proteoglycan fractions from pig laryngeal cartilage*

Fractions are as shown in Scheme 1. Each value given is the average of two separate analyses on duplicate hydrolysates. No corrections were applied for losses during hydrolysis. Cysteine, methionine and tryptophan were determined separately as described in the text.

		Composition (residues/1000 residues)						
Amino acid	Fraction ...	ND1	D1-1	D1-2	D1-3	D1-4	D1-5	D1-6
Asp		78	77	78	78	81	79	88
Thr		58	58	58	59	59	60	63
Ser		128	123	124	122	117	111	98
Glu		147	145	149	151	148	141	141
Pro		89	92	88	84	83	83	83
Gly		144	136	135	127	126	123	112
Ala		73	77	77	78	80	81	84
Val		67	69	69	73	71	68	72
Ile		38	38	38	40	40	41	37
Leu		77	78	74	79	81	81	79
Tyr		15	16	15	17	20	19	21
Phe		24	27	26	28	30	32	33
His		14	8	11	9	9	10	10
Lys		9	16	18	17	15	18	23
Arg		35	41	38	39	38	42	50
Cys		10	6	5	5	—	7	—
Met		10	9	10	10	—	9	8
Try		6	8	9	8	8	7	9

Table 6. *Analysis of subfractions of fraction ND1 after gel chromatography on Sepharose 2B in the presence of* 1.0% *(w/w) hyaluronate as described in the text*

Fractions were as shown in Fig. 2(*b*). Protein content is from summation of amino acid analyses.

	Galactosamine	Hexuronate	Cysteic acid
Fraction	glucosamine (molar ratio)	protein (wt. ratio)	(residues/1000 amino acid residues)
ND1-A	—	5.27	10
ND1-B	32.0	5.88	3
ND1-C	37.2	4.35	3

When the reduced proteoglycan was re-oxidized, after removal of the dithiothreitol by pressure dialysis under O_2 up to 87% of the initial viscosity of the proteoglycan–hyaluronate complex was regained, suggesting that the disulphide bridge(s) necessary for the conformation of the binding site had re-formed. However, under these conditions of pH, ionic strength and temperature the protein is unlikely to have been completely unfolded by reduction, and other charge interactions, hydrophobic interactions and hydrogen bonds may have maintained sufficient structure to enable the correct disulphide bridges to be re-formed. Reduced disulphide bridges were also re-oxidized in the absence of hyaluronate, with a recovery of up to 77% of the hyaluronate-binding

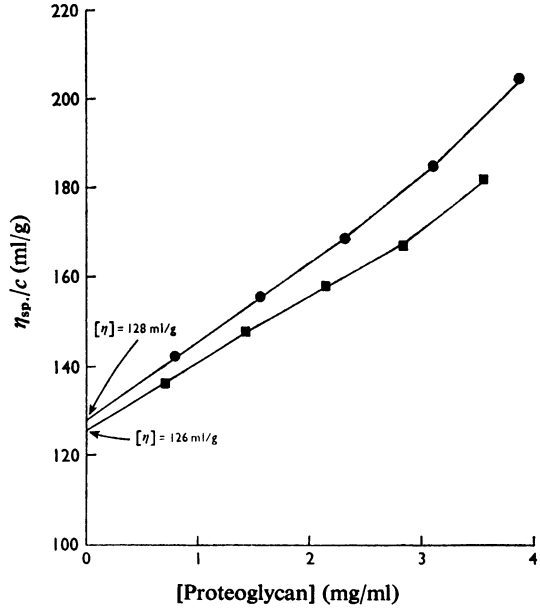


Fig. 2. *Determination of the limiting viscosity number,* [η], *of disaggregated proteoglycan (fraction D1) before* (●) *and after* (■) *reduction and alkylation*

The proteoglycan was in 0.5M-guanidinium chloride/0.05M-sodium acetate, pH5.8, and the viscosity was measured with an Ostwald capillary viscometer at 30°C.

capacity. Although the recovery was less complete than in the presence of hyaluronate, its presence was obviously not essential for the correct disulphide bridges involved in the structure of the binding site to re-form.

Chemical modification of cationic groups on the proteoglycan. The pK_a of the carboxyl group of the glucuronate residues of the hyaluronate is 3.21 (Laurent, 1970). Therefore over the pH range at which binding takes place, pH4–9 (Hardingham & Muir, 1972*a*), the carboxyl groups are fully ionized and are probably involved in interaction with cationic groups such as lysine or arginine residues at the binding site of the protein core. To investigate which of these residues might be involved, various protein-modification reactions were carried out on the proteoglycan.

(*a*) Reaction with acetic anhydride. The proteoglycan was acetylated with 0.53M-acetic anhydride, pH8.5, in either 0.1M-borate buffer or half-saturated sodium acetate. Gel chromatography on Sepharose 2B showed that after reaction the hydrodynamic size of the proteoglycan was essentially unaffected, but that the proteoglycan no longer interacted with added hyaluronate. Determination of free amino groups on the proteoglycans with 2,4,6-trinitrobenzenesulphonic acid (Habeeb, 1967) showed the loss of 40% in borate buffer, whereas 90% were lost in half-saturated sodium acetate. The groups acetylated would include ε-amino groups of lysine, *N*-terminal amino groups and any non-acetylated hexosamine residues. Although acetylation of tyrosine residues cannot be ruled out, the formation of *O*-acetyltyrosine is not favoured in half-saturated sodium acetate (Riordan & Vallee, 1967) when the loss of binding was greatest. Reaction with lower concentrations of acetic anhydride (53mM or 5.3mM) showed very little loss of binding, although 26% and 18% respectively of amino groups were acetylated under these conditions (Table 7). The loss of binding as a result of reaction with acetic anhydride thus involved the blocking of at least 40% of the free amino groups on the proteoglycan.

(*b*) Reaction with 2-methylmaleic anhydride. Although reaction with acetic anhydride suggested that lysine groups might be involved in binding to hyaluronate, other changes in protein structure were possible. The effect of substitution of amino groups with 2-methylmaleic anhydride (citraconic anhydride) was therefore examined, since it has greater specificity for free primary amino groups (Dixon & Perham, 1968). Treatment as described by Dixon & Perham (1968) with 0.55M-citraconic anhydride resulted in complete loss of binding capacity without change in the gel-chromatographic behaviour of the proteoglycan. Attempts to remove the 2-methylmaleyl groups at pH3.0 overnight at 20°C resulted in up to 60% recovery of binding capacity. This substantial recovery of binding showed that no irreversible changes in the conformation necessary for binding had taken place and showed more conclusively that the *N*-terminal amino groups or ε-amino groups of lysine residues were involved in the interaction with hyaluronate. Reactions under similar conditions but with lower concentrations of 2-methylmaleic anhydride showed a significant loss of binding to hyaluronate with 55mM reagent, but no effect with 5.5mM reagent (Table 7).

(*c*) Reaction with butane-2,3-dione (diacetyl). There appeared to be more arginine residues in the hyaluronate-binding region than elsewhere in the protein core. To test whether these contributed to the cationic groups involved in the interaction with the hyaluronate, proteoglycans were treated with 0.5M-butane-2,3-dione in 0.5M-sodium borate, pH7.5, as described by Grossberg & Pressman (1968). This resulted in complete loss of binding to hyaluronate as shown by gel chromatography, and amino acid analysis showed that all the arginine residues had been modified, but there was also some loss of histidine amino acids under these conditions. The reaction was therefore carried out for a shorter time (15min) and at lower temperature (4°C). This resulted in the loss of 29% of the arginine residues, but 85% of the proteoglycan still bound to hyaluronate. The effect of varying the concentration of butane-2,3-dione on the reaction was then examined, the reaction time being kept at 60min. The results (Table 7) showed that the minimum concentration of butane-2,3-dione that would abolish most of the binding was 12mM, and this involved the loss of 89% of the arginine residues. With 5.8mM reagent there was negligible decrease in binding, even though 64% of the arginine residues were modified. Thus under these conditions the alteration of a large proportion

Table 7. *Effect of specific reagents for the modification of amino acid residues on disaggregated proteoglycan (D1)*

Conditions were as described in the text.

Reagent	Concentration (mM)	Binding to hyaluronate (% of control)
Acetic anhydride	5.3	91
	53.0	85
	530	0
2-Methylmaleic anhydride	5.5	100
	55.0	33
	550	0
Butane-2,3-dione	5.8	100
	12.0	5
	58.0	0
2-Nitrophenylsulphenyl chloride	1.0	18

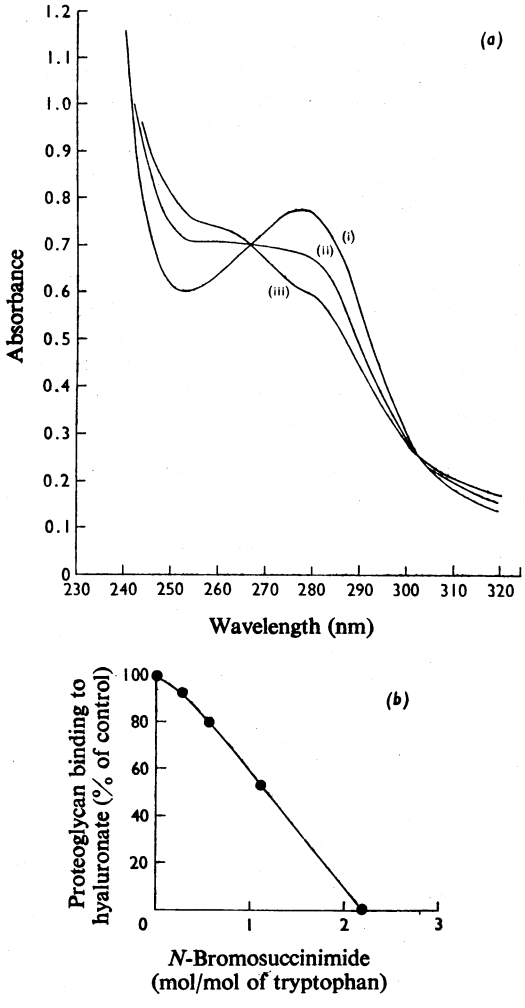


Fig. 3. (*a*) *Effect of N-bromosuccinimide on the u.v. spectrum of disaggregated proteoglycan (fraction D1) and* (*b*) *binding of proteoglycan to hyaluronate after reaction with increasing amounts of N-bromosuccinimide*

The spectra of samples of proteoglycan (10mg/ml) were measured in 0.1M-sodium acetate, pH4.0, with (i) no *N*-bromosuccinimide, (ii) 1.1mol of *N*-bromosuccinimide/mol of tryptophan, (iii) 2.2mol of *N*-bromosuccinimide/mol of tryptophan. The binding to hyaluronate was determined in 0.5M-sodium acetate, pH6.8, by gel chromatography on Sepharose 2B, as described in the text.

of the arginine residues was without effect on the binding of proteoglycan to hyaluronate.

There was no evidence from amino acid analysis for any modification of lysine residues in these experiments, to investigate this further, the reaction of proteoglycan with butane-2,3-dione was also carried out in 0.1M-sodium phosphate, pH6.0, for 6h at 4°C in the dark, conditions which should be even less likely to modify lysine residues (Yankeelov, 1970). This resulted in an 87% loss of binding, and as only 45% of the arginine residues were modified, the conditions appeared to be slightly more selective for those arginine residues that were critical to the binding than was the reaction in borate buffer. There was also no loss of lysine or histidine residues after reaction under these conditions. An apparent loss of tyrosine residues after reaction under all conditions was corrected by adding phenol to the sample before acid hydrolysis (Yankeelov, 1970). The loss of binding on reaction of proteoglycan with butane-2,3-dione thus involved the modification of about 20–28 residues of arginine per molecule.

Modification of tryptophan residues. The tryptophan content of the proteoglycans was quite low, but tryptophan is a particularly hydrophobic amino acid residue and it was therefore likely that some residues would form part of the hyaluronate-binding region if this has a globular structure. The hyaluronate-binding capacity of the proteoglycan was therefore examined after treatment with two reagents that react with tryptophan.

(*a*) Reaction with *N*-bromosuccinimide. If present in excess, *N*-bromosuccinimide reacts with various amino acid residues and results in cleavage of the polypeptide chain (Ramachandran & Witkop, 1967). However, at low concentrations it oxidizes the indole nucleus of tryptophan to oxindole without cleavage of the polypeptide chain. This reaction was followed spectrophotometrically (Fig. 3*a*) and the amount of *N*-bromosuccinimide required to give complete oxidation was determined from the maximum ΔE_{280} (Spande & Witkop, 1967). Calculation of the amount of tryptophan in the proteoglycan from the amount oxidized, assuming the number-average molecular weight of the proteoglycan to be 1.63×10^6, gave about 15 residues per molecule or about 7.5 residues per 1000 amino acid residues, compared with eight residues/1000 residues determined by amino acid analysis. The amount of *N*-bromosuccinimide used was only 2.5mol/mol of tryptophan, which should have minimized reaction with other amino acid residues (Spande & Witkop, 1967). Gel chromatography of proteoglycan that had been treated with 10–200% of that required for maximum ΔE_{280} showed no evidence of peptide cleavage, although cleavage was evident at 25mol of *N*-bromosuccinimide/mol of tryptophan. Determination of the capacity of these modified proteoglycans to bind to hyaluronate showed a direct correlation between the extent of the spectral change and the loss of binding (Fig. 3*b*), which suggested that the tryptophan residues involved in maintaining the necessary conformation of the binding site were not more or less sensitive to oxidation than the average.

(*b*) Reaction with 2-nitrophenylsulphenyl chloride.

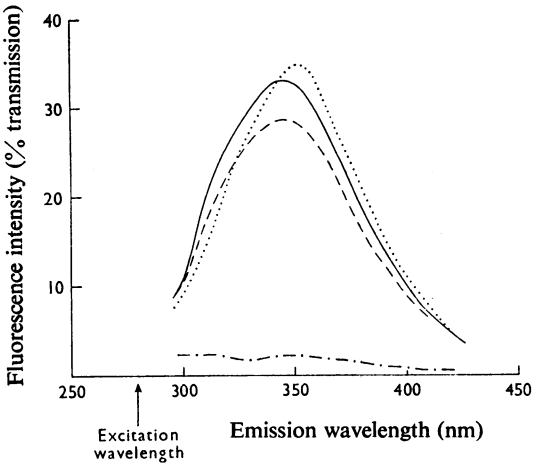


Fig. 4. *Fluorescence of disaggregated proteoglycans*

Fluorescence measurements were made in 0.1M-sodium phosphate, pH6.8, of disaggregated proteoglycan (fraction D1) (——), fraction D1 in the presence of 1% (w/w) hyaluronate (– – – –), fraction D1 in the presence of 0.1% sodium dodecylsulphate (· · · ·) and fraction D1 after reaction with nitrophenylsulphenyl chloride as described in the text (–·–·).

The introduction of a thioether group at position 2 of the indole nucleus by 2-nitrophenylsulphenyl chloride is a fairly specific reaction for tryptophan (Scoffone *et al.*, 1968). The reaction was carried out in 37.5% (w/v) acetic acid, and there was no effect on the molecular size of the proteoglycan, but 82% of its ability to bind hyaluronate was lost. Amino acid analysis showed no change in composition of the acid-stable amino acids. The u.v. spectrum showed a new band at 365nm and enhanced absorption at 280nm. From the increase in absorption at 365nm it was calculated that 5.6mol of tryptophan/mol of proteoglycan (about 35%) was modified by reaction with 2-nitrophenylsulphenyl chloride.

The results of the reaction of proteoglycan with minimal amounts of *N*-bromosuccinimide and with 2-nitrophenylsulphenyl chloride suggested that tryptophan residues were involved in interactions essential for the maintenance of the binding activity of the proteoglycan. Amino acid analysis revealed no significant loss of other acid-stable amino acids. The fluorescence of tryptophan (see below) was decreased by reaction with both reagents.

Fluorescence of proteoglycan

The natural fluorescence of the proteoglycan was examined at various excitation wavelengths in the u.v. region of the spectrum. There was only a single major emission band in the spectrum, which was characteristic of tryptophan with a maximum fluorescence at 346–348nm and an excitation optimum at 280nm (Fig. 4). As it was possible that tryptophan residues close to the hyaluronate-binding site of the molecule would be subject to perturbation when binding took place the spectrum was examined before and after the addition of hyaluronate to proteoglycan under binding conditions. However, there was no major change in the wavelength of the fluorescence maximum and only a slight fall (about 10%) in the intensity. There was thus no evidence of major conformational changes affecting tryptophan residues, and the slight change observed did not form a useful basis for measuring the binding between proteoglycan and hyaluronate.

The fluorescence of proteoglycan was also examined in 0.1% sodium dodecyl sulphate, which abolishes binding to hyaluronate (T. E. Hardingham & H. Muir, unpublished work) and should disrupt the tertiary structure of the binding region. The fluorescence maximum was shifted to longer wavelength (350–355nm), which would correspond to the tryptophan residues being in a more polar phase (Brand & Witholt, 1967) (Fig. 4). This effect was also observed with reduced and alkylated proteoglycan, the fluorescence maximum of which was at longer wavelength (350–355nm). In neither case was the intensity of fluorescence significantly altered.

The results suggested that tryptophan residues did not form direct subsite interaction with hyaluronate, but that there was some evidence of a shift into a more polar environment on denaturation with sodium dodecyl sulphate or after reduction and alkylation of cysteine residues, which was to be expected if some tryptophan residues in the native state formed the internal part of a globular protein structure.

Discussion

Aggregating proteoglycans

The results of the subfractionation of proteoglycans extracted with 0.15M-NaCl and 4M-guanidinium chloride confirmed and extended previous observations (Hardingham & Muir, 1974). Among the aggregating proteoglycans there was a large variation in protein and uronic acid content, which can be interpreted as reflecting a large difference in the degree of substitution of the protein core with chondroitin sulphate chains (Hardingham & Muir, 1974). The relative contents of protein and keratan sulphate were much less variable in proteoglycans of different chondroitin sulphate content. The sequence of addition of the different glycosaminoglycan chains to the protein core is unknown, but the small variation in keratan sulphate content suggests that it may be synthesized largely before the addition

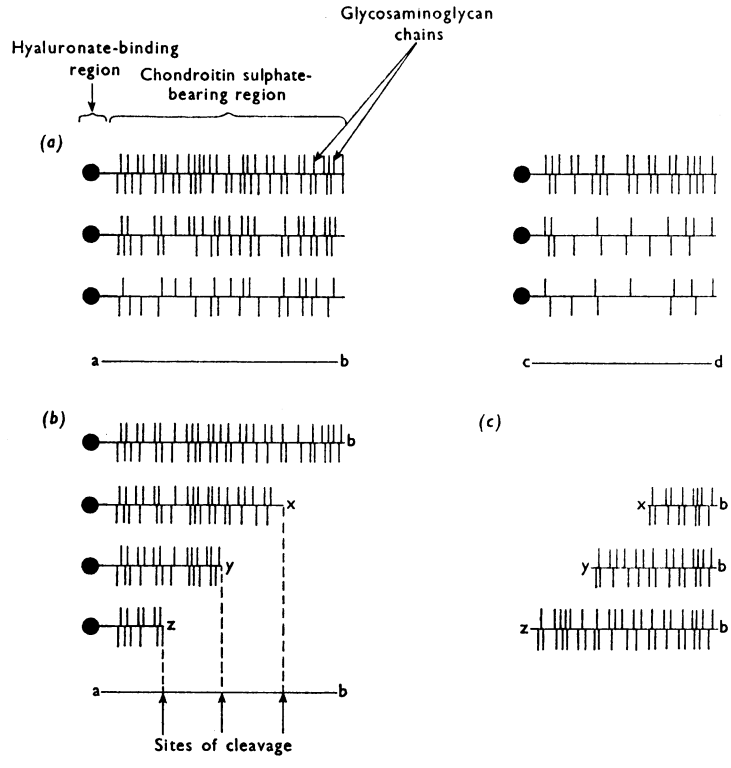


Fig. 5. *Models of proteoglycan structure*

(*a*) Population comprising at least two genetically distinct proteoglycan types, based on polypeptides a–b and c–d, each of constant protein structure but with a variable degree of substitution with glycosaminoglycan chains. (*b*) Population of a single proteoglycan type based on a polypeptide a–b, with a hyaluronate binding region of constant composition and chondroitin sulphate-bearing region of variable polypeptide length. The variable length may arise from cleavage at x, y or z on the polypeptide a–b. (*c*) Non-aggregating proteoglycans of low protein content, deficient in hyaluronate-binding region, possibly derived from the chondroitin sulphate-bearing region of aggregating proteoglycans by cleavage at points x, y or z as in (*b*).

of chondroitin sulphate begins. The variation in the amino acid composition of the different proteoglycan fractions showed that the protein cores were not of uniform structure. This might reflect the presence of proteoglycans based on two or more distinct protein cores (Fig. 5*a*) that overlap each other in their distribution in the density gradient, or there may be only one basic protein core involved that has a variable region in its structure (Fig. 5b).

Some evidence for a variable structure has been provided by the results of Heinegård & Hascall (1974*b*). They have proposed that the proteoglycan protein core is of variable length, since the size distribution of groups of chondroitin sulphate chains released from proteoglycans by exhaustive trypsin/chymotrypsin digestion was very similar in all proteoglycan fractions, irrespective of chondroitin sulphate content. The chondroitin sulphate chains therefore mainly occurred in groups linked by trypsin/chymotrypsin-resistant peptides and the groups contained similar numbers of chondroitin sulphate chains in all the proteoglycans. It was suggested that this reflected a structure which contained two regions (Fig. 5*b*), a hyaluronate-binding site of constant size, which other results (Heinegård & Hascall, 1974*a*) showed was about 90000mol.wt., and a region of variable length of about 110000mol.wt. bearing chondroitin sulphate chains, possibly made up of a repeating polypeptide unit. This would be an important structural feature to establish, as all the protein in the protein core appears to be part of a single covalent structure. Moreover, there is no precedent for the synthesis of a polypeptide with repeating units but of variable length (i.e. from

mRNA of variable length) or for polypeptides being synthesized and subsequently linked together (other than by disulphide bridges). Therefore more probably the region containing chondroitin sulphate could vary in length as a result of partial cleavage after attachment of glycosaminoglycan chains (Fig. 5*b*).

The continuous variation in amino acid composition in proteoglycans of different buoyant density is compatible with such a proteoglycan population of variable structure, and this can be supported by a simple calculation. Serine is the amino acid that varies most in content in different proteoglycan fractions (Table 5). Fractions ND1-B and ND1-C largely lack the hyaluronate-binding site, and hence the serine content of 164 residues/1000 may be similar to that of the region bearing chondroitin sulphate chains, whose average mol.wt. is 110000. The whole disaggregated proteoglycan contains 120 residues of serine/1000 residues, which is an average of the region bearing chondroitin sulphate chains and the hyaluronate-binding region, whose mol.wt. is 90000. From these values, after subtraction of the serine corresponding to the chondroitin sulphate-bearing region, the hyaluronate-binding region is left with 66.2 residues of serine/1000. Assuming the length of the polypeptide-bearing chondroitin sulphate chains to be proportional to the chondroitin sulphate content as in Fig. 5(*b*), then in proteoglycans with 2.5–3 times the protein content of the average, such as fraction D1-6 (Table 3) with a normal hyaluronate-binding region (mol.wt. 90000), the chondroitin sulphate-bearing region should be much shorter (mol.wt. 20200–25400), which would result in a serine content of 84–88 residues/1000 residues. This is considerably lower than the observed value of 98 residues/1000 residues (fraction D1-6, Table 5), which would correspond to a molecule having a much longer chondroitin sulphate-bearing region (mol.wt. 44000). Thus although the serine analyses were compatible with a decrease in size of the chondroitin sulphate-bearing region in the proteoglycans of high protein content, this decrease was less than predicted, which suggested that there was also a lower extent of substitution with chondroitin sulphate chains in these molecules.

Such a hypothetical structure has yet to be confirmed by electron microscopy, whereby the length of the chondroitin sulphate-bearing region could be measured directly. In an electron-microscopic survey of disaggregated proteoglycans from bovine nasal septa, bovine trachea and guinea-pig costal cartilage (Thyberg *et al.*, 1975) the length of molecules was found to vary only within a fairly narrow range (240–360nm), whereas if the chondroitin sulphate-bearing region is assumed to be of constant composition, a threefold change in the weight ratio of chondroitin sulphate to protein would give a decrease in length of the chondroitin sulphate-bearing region from 400nm to 100nm. Since only the region bearing chondroitin sulphate chains is seen in electron micrographs (Thyberg *et al.*, 1975), the variation observed does not give support to the structure proposed in Fig. 5(*b*). However, the proportion of molecules of low chondroitin sulphate content is fairly small and it is possible that some selection may have taken place in preparing the electron micrographs, so that only longer molecules were seen. As the electron micrographs showed no significant variation in the number of groups of chondroitin sulphate chains attached per unit length of chondroitin sulphate-bearing region in molecules of different length, the results also give no support to an alternative structure in which the proteoglycan protein core is of constant length, but has a variable number of chondroitin sulphate chains attached to it. However, the number of chains in each group cannot be measured in the electron micrographs, so further evidence is required before either model (Fig. 5*a* or 5*b*) can be established as a structural feature of proteoglycans from these hyaline cartilages. Measurement of the length of proteoglycans in preparations of intact aggregates may yield the required information, as it was found that the proteoglycans varied in length from 400nm to 100nm in preparations from bovine articular cartilage (Rosenberg *et al.*, 1975), where the proteoglycans are of smaller average size and higher protein content than in hyaline cartilage (Rosenberg *et al.*, 1973).

Hyaluronate-binding region

The proteoglycan-binding site has a high affinity for a decasaccharide unit of hyaluronate (Hardingham & Muir, 1973; Hascall & Heinegård, 1974*b*), and even after the removal of the non-reducing terminal glucuronate residue there was strong binding (Hascall & Heinegård, 1974*b*). By contrast, octasaccharides and smaller fragments could only bind weakly (Hardingham & Muir, 1973; Hascall & Heinegård, 1974*b*). Effective binding is thus to a unit which has at least four carboxyl groups and five acetamido side chains, and this may involve direct interaction with the side chains of various amino acids of the binding site. Lysozyme provides an analogy, for this, as it has a binding site for a hexasaccharide [$(GlcNAc\text{-}MurNAc)_3$] in which the main polar groups are also acetamido and carboxyl groups. The forces involved in binding have been elucidated in great detail and involve at least 15 subsite interactions (Chapman & Sharon, 1969), and there are likely to be as many, if not more, subsite interactions involved in binding hyaluronate to proteoglycan.

The results of various chemical modifications of amino acid residues of the proteoglycan must be treated with some caution, because the specificity of the reagents in most cases is not absolute and the

possibility of side reactions affecting a few critical residues cannot be ruled out, particularly since the mol. wt. of the proteoglycan protein core is estimated to be about 200000 (Hascall & Sajdera, 1970), corresponding to about 2000 amino acid residues, and amino acid analysis would not be sufficiently sensitive to show the loss or modification of one or two residues per molecule.

Among the reactions investigated, the substitution of amino groups with 2-methylmaleic anhydride could be reversed with the recovery of 60% of the binding activity. This showed that amino groups were very important to the functional structure of the binding region and it appears likely that some amino groups may take part in direct subsite interactions with the carboxyl groups of hyaluronate. Acetylation likewise blocked binding, except when a low concentration of acetic anhydride was used (Table 5). Mild acetylation of the intact proteoglycan aggregate did not affect binding (Heinegård & Hascall, 1974*a*), but in this case those amino groups necessary for binding may have also been protected by the presence of hyaluronate and protein link. Reaction with butane-2,3-dione suggested that arginine residues may also be involved in subsite interactions.

The structure of the hyaluronate-binding site appeared to be fairly sensitive to chemical modifications, since a variety of reagents under mild conditions abolished the ability to bind hyaluronate. Nevertheless, since the effects of (*a*) reduction of disulphide bridges and (*b*) substitution of amino groups with 2-methylmaleyl groups could be reversed, the native structure of the binding site appears to be thermodynamically preferred and of intrinsic stability under the conditions of ionic strength, pH and temperature used in these experiments.

The ease of chemical modification is in contrast with the remarkable thermal stability of the hyaluronate-binding site of the unmodified proteoglycans, which was virtually unchanged by heating to 60°C (Hardingham & Muir, 1975), whereas at 80°C the half-life was 148 min and at 100°C, 14.2 min. Although these results were obtained in 0.5M-guanidinium chloride/0.05M sodium acetate, pH 5.8, comparable results were also obtained in 0.15M-NaCl at neutral pH. The resistance to thermal denaturation may be related to the relatively slow metabolic turnover of cartilage proteoglycans. Estimates of their half-lives vary from weeks (Davidson & Small, 1963) to several months (Maroudas, 1974) and are much longer than those for most intracellular proteins. The high resistance to thermal denaturation may be related to the ability of the proteoglycan to retain an active binding site for up to several months *in vivo* without denaturation.

The location of the groups modified was not investigated in the present study, but as the analyses showed that no amino acid residues were exclusive to either the hyaluronate-binding region or the chondroitin sulphate-bearing region of the molecule, they would be distributed in both regions. The relative reactivity of residues in different parts of the protein core will depend on their location and the degree of protection or enhancement given them by neighbouring groups. In general, amino acid residues that form the interior of a globular structure are less reactive to reagents in aqueous solution than those on the surface (Glazer, 1970). Those amino acid residues in the extended polypeptide of the chondroitin sulphate-bearing region may thus be modified more readily than those in the interior of the hyaluronate-binding region. Many of the residues modified may thus be remote from the binding site and without effect on it. Although it appeared that quite a high proportion of arginine, lysine or tryptophan residues were modified before the loss of binding was complete, this indicated only the upper limit of the number of residues directly involved in maintaining the stability of the binding site.

The present results suggest that the hyaluronate-binding region of the molecule is a fairly complex structure that is maintained in a native state by five to seven intermolecular cystine bridges per molecule and various interactions between amino acid side chains. Measurement of the intrinsic tryptophan fluorescence supported the view that the binding region was globular in structure, and as there was no change in fluorescence on addition of hyaluronate to proteoglycan, tryptophan residues did not appear to be involved in direct subsite interactions with the hyaluronate.

In addition to the disulphide bridges, binding to hyaluronate probably depended on the integrity of ε-amino groups of lysine, arginine residues and tryptophan residues.

Non-aggregating proteoglycans

Proteoglycans extracted in 0.15M-NaCl were previously shown to be of comparatively small average size and low protein and keratan sulphate content and were unable to bind to hyaluronate (Hardingham & Muir, 1974). The amino acid composition suggested that the hyaluronate-binding region was incomplete or absent from these proteoglycans. As the binding region is probably globular, and is unlikely to contribute significantly to the hydrodynamic size of proteoglycans, the extensive polydispersity of this fraction suggests that either there was a large variation in chondroitin sulphate substitution and/or the polypeptide backbone was of variable length.

It is possible that proteoglycans extracted in this way are degradation products, but there is some evidence against this. If they result from the action of

proteinases on intact proteoglycans during extraction, the proportion of proteoglycans extracted with 0.15M-NaCl should increase with the time of extraction, whereas the yield did not increase very much beyond 3h at 4°C (Hardingham & Muir, 1974). It has been shown that the action of neutral proteinase(s) during the extraction of proteoglycans from a rat chondrosarcoma was inhibited with EDTA, 6-aminohexanoic acid and benzamidine hydrochloride (Oegema *et al.*, 1975). When added to 0.15M-NaCl for the extraction of cartilage at 4°C, they slightly increased the yield of the proteoglycans, but did not significantly increase the proportion that was able to bind to hyaluronate (T. E. Hardingham & H. Muir, unpublished work). This fraction was therefore unlikely to be an artifact of the preparation, although it could be a product of the natural breakdown of proteoglycan *in vivo*. On the other hand, the incorporation of [^{35}S]sulphate into proteoglycans of different size showed that smaller molecules were labelled more rapidly than larger ones (Hardingham & Muir, 1972*b*). Moreover, in pulse–chase experiments, no shift in the distribution of radioactivity occurred between fractions with time, which suggests that all fractions were synthesized simultaneously (Hardingham & Muir, 1972*b*) and the small non-aggregating proteoglycans were formed in parallel with those that aggregate. The non-aggregating proteoglycans may be a distinct molecular species, but if the polydispersity in the protein/core of aggregating proteoglycans arises as the result of enzymic cleavage of the chondroitin sulphate-bearing region before leaving the cell, they may be fragments of the chondroitin sulphate-bearing region cleaved from aggregating proteoglycans, as shown in Fig. 7(*c*).

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