Proteoglycans of Hyaline Cartilage

ELECTRON-MICROSCOPIC STUDIES ON ISOLATED MOLECULES

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Proteoglycan monomers from guinea-pig costal cartilage, bovine nasal and bovine tracheal cartilage were observed in the electron microscope after being spread in a monomolecular layer with cytochrome *c*. The proteoglycan molecule appeared as an extended central core filament to which side-chain filaments were attached at various intervals. The molecules from the three sources displayed great ultrastructural similarities. On average, the core filament was about 290 nm long, there were about 25 side-chain filaments per core filament, the side-chain filaments were about 45 nm long, and the distance between the attachment points of the side-chain filaments to the core filament was about 11 nm. With regard to overall size of the molecules, no evidence of distinct subpopulations was obtained. Good correlation was found between ultrastructural data for the proteoglycan molecules and chemical data obtained by enzyme digestions and gel chromatography. Together these data strongly support the interpretation of the electron-microscopic pictures as indicating a central filament corresponding to the protein core and side-chain filaments corresponding to the chondroitin sulphate chain clusters of the proteoglycan monomers.

The proteoglycans of cartilage are complex molecules with several levels of organization. First, chondroitin sulphate and keratan sulphate chains are covalently linked to a protein core, forming a polydisperse population of proteoglycan monomers. Secondly, these monomers aggregate by interaction with hyaluronic acid and link proteins to form very large complexes, i.e. proteoglycan aggregates (Hardingham & Muir, 1972; Gregory, 1973; Hascall & Heinegård, 1974a). *In vivo* the proteoglycans probably occur in such aggregates to a large extent (Hascall & Heinegård, 1974a).

Although a great deal of information on the structure of proteoglycans has been obtained from biochemical and biophysical studies (Hascall & Sajdera, 1969, 1970; Pasternack *et al.*, 1974; Hascall & Heinegård, 1974a,b; Heinegård & Hascall, 1974a,b), a great many questions still remain unanswered, such as the degree of polydispersity and heterogeneity at the proteoglycan-monomer level. To some extent, this may be due to shortcomings in the analytical methods normally used in these investigations. Electron microscopy was therefore introduced by Rosenberg *et al.* (1970) as a complementary technique for the study of isolated proteoglycans in order to observe and measure individual molecules. A similar technique was subsequently used by Wellauer *et al.* (1972). Electron microscopy has also been used to observe hyaluronic acid (Fessler & Fessler, 1966) and heparin (Hirano,

1972). Slightly modified versions of the preparative method of Kleinschmidt & Zahn (1959), originally devised for DNA, were used in these studies. In this technique, polyanionic macromolecules (e.g. proteoglycans) are mixed with a basic protein, usually cytochrome *c*, and the protein–polyanion complexes are spread at an air/liquid interface. The resulting film can then be picked up on a grid, stained with uranyl acetate and/or shadowed with heavy metals, for examination in the electron microscope.

This report describes an electron-microscopic investigation of proteoglycan monomers isolated from three different hyaline cartilages: guinea-pig rib, bovine nasal and bovine tracheal cartilage. A simplified droplet technique, modified from that of Lang & Mitani (1970), was used to prepare cytochrome *c*–proteoglycan films. Qualitative and quantitative data are presented which partly confirm and partly extend the data obtained by Rosenberg *et al.* (1970) and Wellauer *et al.* (1972). The results are discussed in relation to current theories about the macromolecular structure of proteoglycans.

A preliminary account of part of this work has been published (Lohmander & Thyberg, 1975).

Materials and Methods

Unless otherwise stated, all chemicals used were of analytical grade. Water was redistilled in glass.

Isolation of proteoglycans

Proteoglycans were isolated from guinea-pig costal cartilage and from bovine nasal and tracheal cartilage by extraction with 4M-guanidine hydrochloride, pH 5.8 (Sajdera & Hascall, 1969), and purified by using CsCl-density-gradient centrifugation under associative and dissociative conditions (Hascall & Sajdera, 1969). The material used for electron microscopy was obtained from previous studies (Hascall & Heinegård, 1974a; Lohmander, 1975). The bottom fraction of the dissociative centrifugation, A1-D1 [Heinegård's (1972) terminology has been used in this paper], was used. This fraction consisted essentially of monomeric proteoglycan molecules. Detailed descriptions of the preparation and characterization of proteoglycans from the three cartilages have been given elsewhere (Heinegård, 1972; Hascall & Heinegård, 1974a; Lohmander, 1975). Chondroitin sulphate peptide fragments, 'clusters', were prepared by trypsin-chymotrypsin digestion of proteoglycan monomers prepared from bovine tracheal cartilage (Heinegård & Hascall, 1974b). The fractions used in this study were numbers 2, 3 and 5, suggested to contain about six, four and one polysaccharide chains respectively, attached to a peptide core fragment.

Characterization of proteoglycans

To determine the size of the proteoglycans, samples (1 mg) were chromatographed on a column (0.8 cm × 145 cm, V_t 85 ml) of Sepharose 2B (Pharmacia Fine Chemicals, Uppsala, Sweden) eluted with 0.5M-sodium acetate, pH 7.0.

To determine the size of the chondroitin sulphate molecules, samples (0.5 mg) of the proteoglycans were digested with 10 µg of crystalline papain (Sigma Chemical Co., St. Louis, Mo., U.S.A.) for 10 h at 65°C in a buffer of 0.05M-sodium phosphate, 0.005M-EDTA and 0.01M-cysteine-HCl, pH 6.5 (Heinegård & Hascall, 1974b), and subsequently chromatographed on a column (0.8 cm × 137 cm, V_t 84 ml) of Sephadex G-200 (Pharmacia) eluted with 0.5M-sodium acetate, pH 7.0.

To determine the size of the chondroitin sulphate clusters, samples of the proteoglycans (1 mg) were digested with 20 µg of crystalline trypsin (Koch-Light Laboratories, Colnbrook, Bucks., U.K.) under toluene for 24 h at 25°C in 0.05M-sodium phosphate, pH 7.6. After addition of 20 µg of an α -chymotrypsin (Koch-Light) digestion was continued for another 48 h at 25°C. All enzymic treatments were performed as described by Heinegård & Hascall (1974b). Sepharose 6B (Pharmacia) chromatography was performed on a column (0.8 cm × 145 cm, V_t 81 ml) eluted with 0.5M-sodium acetate, pH 7.0.

Column effluents were analysed for uronic acid contents with a Technicon autoanalyser as described by Heinegård (1973).

Preparation for electron microscopy

Proteoglycans were dissolved at 4°C overnight at a concentration of 0.1 mg/ml in a buffer of 0.10M-KCl in 0.05M-potassium phosphate, pH 7.0 (Rosenberg *et al.*, 1970). The stock solutions obtained were kept frozen in 0.2 ml portions before the experiments. The method used for spreading the proteoglycans was a modification of the procedure devised for DNA by Lang & Mitani (1970). Solutions containing 0.5 µg of the different proteoglycan fractions/ml and 1–10 µg of cytochrome *c* (Sigma, type VI)/ml in 0.01, 0.05 or 0.3M-ammonium acetate, pH 5.0, were prepared at 23–25°C. Droplets (50 µl) of the spreading solutions were deposited on a plate of Teflon and covered by an inverted Petri dish. The spontaneously formed cytochrome *c*-proteoglycan film was transferred after 30 min to a grid coated with carbon-stabilized Formvar film by touching the grid to the droplet surface. The grids were air-dried after thorough rinsing in double-distilled water. They were stained with 0.1 mm-uranyl acetate in acetone for 1 min, rinsed in acetone and allowed to dry. In some preparations the grids were further contrasted by rotary shadowing with platinum at an angle of about 10° and a distance of about 10 cm, by using an Edwards vacuum-coating unit (Edwards High Vacuum, Manor Royal, Crawley, Sussex, U.K.). Alternatively, they were shadowed with platinum-palladium alloy (80:20) by using the same technique without prior uranyl acetate staining.

Electron microscopy and measurements

The grids were examined in a Philips EM 300 electron microscope operated at 60 kV and equipped with an anti-contamination device cooled with liquid N₂. Micrographs were made on Kodak electron-microscope film (no. 4489, 8.3 cm × 10.2 cm) at magnifications of about 15000–60000. The magnifications were repeatedly calibrated with a carbon replica of a ruled diffraction grating having 2160 lines/mm (Ladd Research Industries, Burlington, Vt., U.S.A.). In experiments designed to determine numbers and lengths of different structures in the proteoglycan monomers, grids were scanned without overlapping. Fields containing one or several clearly identifiable proteoglycan molecules with well extended side-chain filaments were photographed at primary magnifications of about 40000. The plates were then enlarged 16–17 times in a projector and the molecules traced on paper (final magnification 600000–700000; to determine the

EXPLANATION OF PLATE I

Electron micrograph of proteoglycan monomers isolated from guinea-pig costal cartilage

Conditions of spreading: 0.01 M-ammonium acetate, pH 5.0, 2 μ g of cytochrome *c*/ml, 0.5 μ g of proteoglycan/ml. Uranyl acetate staining. The bar indicates 300 nm.

EXPLANATION OF PLATE 2

Electron micrograph of proteoglycan monomer isolated from bovine tracheal cartilage

Conditions of spreading: 0.3M-ammonium acetate, pH 5.0, 1.5 μ g of cytochrome *c*/ml, 0.5 μ g of proteoglycan/ml. Uranyl acetate staining and platinum-palladium shadowing. The bar indicates 100nm.

Fig. 1. Schematic drawing of part of a proteoglycan monomer

The principles are indicated for determination of the number of side-chain filaments. The arrows indicate the distances recorded between the attachment points of the side-chain filaments to the core filament. In addition to these distances marked by arrows, a zero was also recorded as distance in case B and C. These zero values are included in the distributions shown in Fig. 6 and in the mean values in Table 1.

exact magnification in the projector a glass-plate replica with a mm scale was used). The following data were recorded from the traced molecules and transferred to punched cards for further processing in a computer: (1) core filament length (nm), (2) number of side-chain filaments per core filament, (3) length of individual side-chain filaments (nm) and (4) distances between consecutive side chains along the core filament, including distances between the end points of the core filament and the outermost attachment points of side-chain filaments (nm). The interpretation of the electron-microscopic pictures is exemplified in Fig. 1. Length measurements were made with a micrometer modified into a map ruler with a scale division corresponding to 1mm on the paper. Thus 1 scale division on the micrometer corresponded to a real distance of about 1.4–1.7nm at the final magnifications used. Distances smaller than 2mm (parameter 4) were made equal to zero.

Statistical methods

Statistical evaluations of the collected data were made in an IBM 360/75 computer by using standard biomedical computer programs (Health Sciences Computing Facility, University of California, Los Angeles, Calif., U.S.A.). The conformity of the distributions of the different parameters to the normal distribution was examined with Kolmogorov's test (Keeping, 1962).

Results and Discussion

Electron microscopy

Regardless of the origin of the material, the ionic strength or the cytochrome *c* concentration of the spreading solution or the method used for contrasting the specimens, the proteoglycan molecules of fraction A1-D1 appeared as an extended central core filament to which side-chain filaments were attached at various intervals (Plates 1 and 2). After uranyl acetate staining, which usually gave the best overall contrast with the molecules, the core filament was frequently difficult to see clearly over its entire length. However, it could be made out fairly well by observing the attachment points of the side chains, although extensions beyond the outermost of these points were frequently difficult to determine. The core became more clearly visible after shadowing, or uranyl acetate staining followed by shadowing. The side-chain filaments were distributed along the entire length of the core and extended from it in a roughly perpendicular direction. They were mainly well separated from one another. However, two or more side chains occasionally appeared to be interlaced. Thus two chains originating independently from the core filament could be seen to merge into a single thicker structure. In other instances one side-chain filament divided into two or more thinner branches. Adjacent chains were also occasionally seen to cross. After spreading at the low ionic strengths, the side chains usually appeared to be fewer in number and more distinct in outline.

The general appearance of the proteoglycan molecules agreed with the description provided by Rosenberg *et al.* (1970) and Wellauer *et al.* (1972) for bovine nasal-cartilage proteoglycans. However, in view of recent chemical data on the proteoglycan structure (Hascall & Sajdera, 1969, 1970; Hascall & Heinegård, 1974a,b; Heinegård & Hascall, 1974a,b) it seems necessary to modify previous interpretations of the fine structural pattern obtained after spreading these molecules into a two-dimensional monomolecular layer, as is discussed below.

A few distinct, normally unbranched strands, varying in length from 20–30nm to 1μm or more, were occasionally seen in spreadings of the A1-D1 preparations (cf. Lohmander & Thyberg, 1975; Fig. 2). These filaments were mostly well separated from the proteoglycan molecules. They conceivably represented either nucleic acid or hyaluronic acid.

Counting and measurement of different components of the proteoglycan monomers were performed after spreading in 0.3M-ammonium acetate at pH5.0 to allow comparison with the results of previous studies (Rosenberg *et al.*, 1970; Wellauer *et al.*, 1972). Quantitative determinations were made

Table 1. *Electron-microscopic measurements of proteoglycan molecules (fraction A1-D1)*

Mean values are given with standard deviations in parentheses.

Source of proteoglycan	Staining	Number of proteoglycan molecules measured	Length of core filament (nm)	Number of side-chain filaments per core filament	Length of side-chain filaments (nm)	Distance between attachment points of side-chain filaments (nm)
Guinea-pig rib cartilage	Stained with uranyl acetate	102	282.8 (38.3)	30 (4.7)	38.6 (12.5)	10.1 (9.2)
Bovine nasal cartilage	Stained with uranyl acetate	101	288.9 (41.2)	25 (4.8)	45.6 (13.3)	12.5 (11.7)
Bovine nasal cartilage	Shadowed with Pt-Pd alloy	100	306.7 (60.4)	23 (4.3)	45.4 (15.2)	13.9 (12.7)
Bovine tracheal cartilage	Stained with uranyl acetate and shadowed with Pt	125	291.3 (43.8)	29 (4.0)	46.1 (11.4)	11.3 (10.9)

on pictures taken from one and the same grid so as to minimize possible causes for artificial variations in the results. However, two grids were used for material isolated from bovine nasal cartilage in order to test the reproducibility of the spreading technique and to evaluate the possible influence of the method used for contrasting the specimens. For this purpose a spreading solution was prepared, and after spreading the grid was stained with uranyl acetate in acetone. The spreading solution was then frozen, thawed 2 days later, and a new droplet spreading was made. This time the grid was shadowed with platinum-palladium alloy, omitting uranyl acetate staining.

Before the quantitative data are considered in any detail, it should be pointed out that the extent to which they are representative of the entire population of proteoglycan molecules is not known for certain. Thus it is not clear whether all the molecules in the A1-D1 fraction are equally well incorporated into the cytochrome *c*-proteoglycan film. Moreover, structures that appeared to represent fragmented or incompletely adsorbed molecules were frequently observed. They were not included for quantitative analyses. There were also many molecules which were too indistinct to permit adequate estimation of the different parameters. However, it is not likely that any systematic exclusion of molecules of any special type(s) would be made by these means.

As shown in Table 1 the two spreadings of bovine nasal-cartilage proteoglycans produced similar results with respect to the structure of the molecules and the dimensions of various parameters. Thus no significant differences (Student's *t* test: $P > 0.05$) were obtained in the mean length of the side-chain filaments. The small but definite ($P < 0.01$) difference in the number of side-chain filaments could have been due to difficulties in distinguishing two or more

closely attached filaments after shadowing with heavy metals, which, compared with uranyl acetate staining, lends a rather coarse granularity to the picture. The increase in the length of the core filament after shadowing ($P < 0.05$), and the concomitant increase in the mean distance between the side-chain filaments along the core ($P < 0.001$), are probably largely ascribable to the fact that the core filament is more clearly observed throughout its length after this type of contrasting. For the sake of simplicity, as far as the proteoglycans isolated from bovine nasal cartilage are concerned, the following discussion will only consider the material stained with uranyl acetate. The results obtained with the shadowed material agree therewith unless otherwise stated.

As further shown in Table 1 the proteoglycans from the three separate sources were ultrastructurally rather similar. However, the mean distance between the attachment points of the side-chain filaments and the mean length of the side-chain filaments was shorter in the rib cartilage molecules ($P < 0.001$) than in the other two materials.

Fig. 2 demonstrates the distributions of core filament lengths. In all three materials the length of the molecules ranged from about 170 to about 370 nm. Kolmogorov's test (Keeping, 1962) revealed that there were no significant deviations from a normal distribution ($P > 0.05$). Thus with regard to the overall size of the molecules no distinct subpopulations appeared to exist among the proteoglycans of the A1-D1 fractions. In this respect, the present results are in contrast with those of Rosenberg *et al.* (1970) and Wellauer *et al.* (1972). In ultrastructural studies on bovine nasal-cartilage proteoglycans, these authors postulated the existence of two molecular species, a short (monomeric) and a long (dimeric) form. However, they presented no valid statistical evidence in support

Fig. 2. Distributions of core filament lengths of proteoglycan monomers, for (a) guinea-pig costal cartilage, (b) bovine nasal cartilage and (c) bovine tracheal cartilage

For details see under 'Electron microscopy and measurements'.

of any such interpretation of their pictures. Further, since these authors used a proteoglycan prepared by disruptive techniques for their investigations, it is possible that degradation of molecules due to high shear forces and/or proteolysis could have occurred during the preparation (Sajdera & Hascall, 1969). In later work on articular-cartilage proteoglycans, L. Rosenberg (personal communication) found no more than one population with respect to core-filament length. These proteoglycans were prepared by the method of Hascall & Sajdera (1969).

The distributions of the number of side-chain filaments per core filament (Fig. 3) did not differ significantly from the normal ($P > 0.05$) in any of the three materials, again contradicting the existence of separate molecular species in the A1-D1 fractions.

The average side-chain filament length per core molecule varied within rather wide limits (Fig. 4). The distributions of these mean values did not differ significantly from the normal distribution. The wide variations in the mean length of side-chain filaments could indicate the existence of proteo-

Fig. 3. Distributions of the number of side-chain filaments per core filament for (a) guinea-pig costal cartilage, (b) bovine nasal cartilage and (c) bovine tracheal cartilage

For details see under 'Electron microscopy and measurements'.

glycan molecules with predominantly short or long side chains respectively at each end of a continuous distribution of molecules. Since a low but significant positive correlation (Table 2) was found between the length of the core filament (variable 1) and the mean length of the side-chain filaments (variable 4) in three of the four measured materials, these results could also be taken as an indication of the existence of short molecules with a predominance of short side-chain filaments and longer molecules with a predominance of longer side-chain filaments. However, it should again be stressed that this change was gradual and that no evidence was obtained supporting the existence of distinct molecular species.

If the side-chain filaments are regarded as separate entities their lengths distribute as shown in Fig. 5. The histograms demonstrate that the distributions are slightly skewed towards longer filament lengths. This asymmetry is probably the reason why, according to Kolmogorov's test, a deviation from the normal distribution occurred in all three materials. Nevertheless, the histograms did not point to the presence of any distinct subpopulations among the side-chain filaments.

The mean distances per core molecule between the side-chain filaments along the core filament displayed rather narrow distributions, which failed to differ significantly from the corresponding normal

Fig. 4. Distributions of average side-chain filament lengths, for (a) guinea-pig costal cartilage, (b) bovine nasal cartilage and (c) bovine tracheal cartilage

For details see under 'Electron microscopy and measurements'.

Fig. 5. Distributions of individual side-chain filament lengths, for (a) guinea-pig costal cartilage, (b) bovine nasal cartilage and (c) bovine tracheal cartilage

For details see under 'Electron microscopy and measurements'.

distributions. Thus more than 90% of these average values for all three materials ranged between 8 and 16 nm. However, when considering the individual distances between consecutive points of attachment to the core filament, strikingly asymmetric distributions were obtained (Fig. 6) with a relatively large proportion of very short distances. It should be pointed out that about 30% of the side-chain filaments were attached to the central core in 'pairs' with a distance between the two chains recorded as zero (case B and C in Fig. 1, representing about 10 and 20% of the number of side chains respectively). If the numbers of side chains in these 'pairs' are recorded as only one, instead of two, and the data are recalculated, each core would contain about 20 side chains with a mean distance along the core of about 14 nm (rib) to 17 nm (trachea, nasal

septum). The distributions of the number of side chains calculated in this way was parallel to those shown in Fig. 3.

Correlation between chemical and electron-microscopic data

Chemical and physicochemical data have shown that proteoglycan monomers (A1-D1 fraction) from bovine nasal cartilage have a central protein core with a molecular weight of about 200 000 (Hascall & Riolo, 1972). A portion of the core, the hyaluronic acid-binding region, is a peptide sequence globular in nature with a molecular weight of about 90 000 (Heinegård & Hascall, 1974a). Therefore the remainder of the core, the polysaccharide-binding region,

Fig. 6. Distributions of distances between attachment points of side-chain filaments to the core filament, for (a) guinea-pig costal cartilage, (b) bovine nasal cartilage and (c) bovine tracheal cartilage

For details see under 'Electron microscopy and measurements'.

should have a molecular weight of the order of 110000. If this part of the protein core were fully extended, it would be about 400 nm long. Early data on proteoglycans have already indicated that the length of the molecules would be of this order of magnitude (Mathews & Lozaityte, 1958).

The chondroitin sulphate side chains, which radiate out from the central protein core, are by far the major polysaccharide component in cartilage proteoglycans. These chains constitute 90–95% of the glycosaminoglycans in cartilage A1-D1 fractions, the remainder consisting of keratan sulphate (Hascall & Sajdera, 1969; Heinegård, 1972; Lohmander, 1975). There are about 100 chondroitin sulphate molecules per protein core in nasal-cartilage

Fig. 7. Elution patterns on Sepharose 2B of proteoglycan monomers (A1-D1) from (a) guinea-pig costal cartilage, (b) bovine nasal cartilage and (c) bovine tracheal cartilage

For experimental details see under 'Characterization of proteoglycans'.

proteoglycans, the number displaying wide variations (Hascall & Sajdera, 1970). The average molecular weight of chondroitin sulphate is about 20000 for nasal cartilage (Wasteson, 1971), i.e. about 40 disaccharide units. Supposing that each disaccharide unit is 0.96 nm long (Atkins *et al.*, 1974), the length of a fully extended chondroitin sulphate chain with $\bar{M}_w$ 20000 would be about 40 nm. However, there is considerable polydispersity, and chondroitin sulphate chains of molecular weights from 3000 to 40000 can be isolated (Wasteson, 1971; Lohmander *et al.*, 1973).

As discussed above, the chondroitin sulphate chains are not distributed symmetrically along the protein core. A large portion of the core, i.e. the hyaluronic acid-binding region, is very probably devoid of chondroitin sulphate chains. Further,

Fig. 8. Elution patterns on Sephadex G-200 of proteoglycan monomers (A1-D1) digested with papain: (a) guinea-pig costal cartilage, (b) bovine nasal cartilage and (c) bovine tracheal cartilage

For experimental details see under 'Characterization of proteoglycans'.

recent data (Mathews, 1971; Heinegård & Hascall, 1974b) indicate that these chains are not distributed evenly along the remainder of the protein core, the chondroitin sulphate-binding region. Results obtained after proteolytic fragmentation of proteoglycans suggested that the chondroitin sulphate chains are attached to the protein in clusters, each cluster containing from one to ten very closely peptide-linked polysaccharide chains, averaging about four chains (Heinegård & Hascall, 1974b). The clusters are believed to be separated by longer peptide sequences. In conclusion, each proteoglycan molecule would contain about 100 chondroitin sulphate molecules linked to protein in about 25 clusters containing a variable number of chains.

The chemical data summarized above and the overall picture of the proteoglycan molecule as a brush-like structure (Mathews & Lozaityte, 1958; Rosenberg *et al.*, 1970) or, expressed as a hydrodynamic model, as an ellipsoid of revolution of low axial ratio (Pasternack *et al.*, 1974), are consistent with the picture seen in the electron microscope. It is likely that the central filament seen in shadowed pictures represents the chondroitin sulphate-binding region of the protein core and that the hyaluronic acid-binding region was not observed with the

Fig. 9. Elution patterns on Sepharose 6B of chondroitin sulphate chain clusters prepared by digestion of proteoglycan monomers (A1-D1) with trypsin-chymotrypsin: (a) guinea-pig costal cartilage, (b) bovine nasal cartilage and (c) bovine tracheal cartilage

For experimental details see under 'Characterization of proteoglycans'.

techniques used. The observed central filament or protein core length of some 300nm is of the same magnitude as would be expected for a rather extended protein core, as discussed above. The number of side chains seen after spreading was 23-30 (Table 1). Since the number of chondroitin sulphate molecules in the proteoglycans is much greater (about 100), it is likely that each side chain ultrastructurally observed represents one of the clusters of polysaccharide chains. The fact that only single filaments with similar overall dimensions to the side-chain filaments of the proteoglycans were observed when preparations of chondroitin sulphate clusters (Heinegård & Hascall, 1974b) were spread provides further support for this interpretation. These fragments appeared as single strands irrespective of whether they originated from fractions containing an average of one, four or six polysaccharide chains per molecule (Heinegård & Hascall, 1974b). It is possible that the chondroitin sulphate chains within a cluster in some cases could spread

Table 2. *Correlation matrix between variables 1-4*

All correlation coefficients are statistically significant at the level $P < 0.01$ or $P < 0.001$ (Student's *t* test). Variable 1, core length (nm); 2, number of side-chain filaments per core filament; 3, average length of side-chain filaments (nm) per core filament; 4, average distance between attachment points of side-chain filaments (nm) per core filament.

Variable	1	2	3	4	Source of proteoglycan	Staining
1		0.467	0.272	0.259	Guinea-pig rib cartilage	Stained with uranyl acetate
		0.526	—	—	Bovine nasal cartilage	Stained with uranyl acetate
		—	0.547	0.563	Bovine nasal cartilage	Shadowed with Pt-Pd alloy
		0.541	0.250	0.488	Bovine tracheal cartilage	Stained with uranyl acetate and shadowed with Pt
2			0.262	-0.488	Guinea-pig rib cartilage	Stained with uranyl acetate
			—	-0.595	Bovine nasal cartilage	Stained with uranyl acetate
			—	-0.478	Bovine nasal cartilage	Shadowed with Pt-Pd alloy
			—	-0.295	Bovine tracheal cartilage	Stained with uranyl acetate and shadowed with Pt
3				—	Guinea-pig rib cartilage	Stained with uranyl acetate
				—	Bovine nasal cartilage	Stained with uranyl acetate
				0.696	Bovine nasal cartilage	Shadowed with Pt-Pd alloy
				—	Bovine tracheal cartilage	Stained with uranyl acetate and shadowed with Pt
4						

in different directions. Such behaviour could explain the fact that some of the side-chain filaments originate from the same point at the central core filament, yielding the 'pairs' discussed above. The two chains within the 'pair' would then represent different parts of the same cluster.

The proteoglycan molecules from the three sources displayed a similar overall ultrastructure, in particular the tracheal and nasal proteoglycans. However, proteoglycans from rib cartilage differed in that they on the average contained shorter side chains (Table 1). The proteoglycans from the three tissues were characterized as described under 'Characterization of proteoglycans' to determine whether or not the ultrastructural data were correlated to chemical data. The elution patterns of A1-D1 proteoglycans on Sepharose 2B displayed only minor differences (Fig. 7), suggesting that these proteoglycans prepared from the three cartilages were of similar hydrodynamic size. Thus the ultrastructural data, which did not display any major differences between the three materials, were consistent with the gel-chromatographic data. The distributions of the size of the chondroitin sulphate molecules in the proteoglycans of the three cartilages did not differ, as measured by Sephadex G-200 chromatography of papain-digested proteoglycans (Fig. 8). Such a result seemingly appears to contradict the significantly shorter average length of the side-chain filaments of the rib-cartilage material, observed ultrastructurally (Table 1). As discussed, however, it is likely that the side-chain filaments seen in the electron microscope correspond to the clusters of chondroitin sulphate molecules, which can be

isolated by trypsin-chymotrypsin digestion of proteoglycans. The Sepharose 6B gel chromatograms shown in Fig. 9 were obtained with A1-D1 proteoglycans digested in this way. It appears that the proportion of low-molecular-weight chondroitin sulphate-peptide fragments is larger in the digest of the rib-cartilage proteoglycans than in the digest of nasal and tracheal proteoglycans. This difference may well explain the shorter average side-chain filament length for rib cartilage proteoglycans, if it is assumed that the length of the ultrastructurally observed side-chain filaments and the hydrodynamic size of the chondroitin sulphate-peptide fragments measure the same molecular parameter. Consistently, there was no difference in the gel-chromatographic patterns of nasal and tracheal A1-D1 trypsin-chymotrypsin digests (Fig. 9), nor any difference in terms of ultrastructural data on the side chains of these proteoglycans (Table 1 and Fig. 5). The variability of all measured ultrastructural parameters agrees with the great polydispersity with respect to protein core and chondroitin sulphate side chains of the proteoglycans determined by chemical methods. In conclusion, the differences observed in the electron microscope appear to be matched by chemical and/or physicochemical differences between corresponding parameters. On the other hand, similarities in ultrastructural parameters match similarities in chemical parameters. Consequently the interpretation of the electron-microscopic pictures as showing a central filament corresponding to the protein core, and side-chain filaments corresponding to the chondroitin sulphate chain clusters, is strongly supported by the chemical data.

Correlation coefficients were calculated between the different ultrastructural variables of the proteoglycan molecules. The results of these calculations are shown in Table 2. There is a positive and significant correlation between the length of the protein core and the number of chondroitin sulphate clusters in all three materials. The molecules from rib and tracheal cartilage also show a positive and significant correlation between the length of the protein core, on the one hand, and the average length of the side chains and the average distance between the side chains along the protein core, on the other hand. In the nasal-cartilage proteoglycans these correlations were found for the shadowed but not for the uranyl acetate-stained molecules. To summarize, the correlation matrices indicate that there is a concurrent increase in the number of chondroitin sulphate clusters per core when the size of the molecules, i.e. the length of the protein core, increases. Moreover, there are indications that the average size of the clusters increases, as well as the average distance between them, when the length of the protein core increases. The interpretation of these latter results in terms of proteoglycan structure is not clear at the present. One possible explanation could be that proteoglycan monomers are built up from several subunits of differing composition, e.g. with respect to the distance between and size of chondroitin sulphate clusters. The relative proportions between these subunits could then change with changing size of the proteoglycans.

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