Biochem. J. (1978) **175**, 913–919
Printed in Great Britain

Cartilage Proteoglycan Aggregates

ELECTRON-MICROSCOPIC STUDIES OF NATIVE AND FRAGMENTED MOLECULES

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(*Received* 14 *April* 1978)

1. Proteoglycan aggregates from bovine nasal cartilage were studied by using electron microscopy of proteoglycan/cytochrome *c* monolayers. 2. The aggregates contained a variably long central filament of hyaluronic acid with an average length of 1037nm. The proteoglycan monomers attached to the hyaluronic acid appeared as side chain filaments varying in length (averaging 249nm). They were distributed along the central filament at an average distance of about 36nm. 3. Chondroitin sulphate side chains were removed from the proteoglycan monomers of the aggregates by partial chondroitinase digestion. The molecules obtained had the same general appearance as intact aggregates. 4. Proteoglycan aggregates were treated with trypsin and the largest fragment, which contains the hyaluronic acid, link protein and hyaluronic acid-binding region, was recovered and studied with electron microscopy. Filaments that lacked the side chain extensions and had the same length as the central filament in the intact aggregate were observed. 5. Hyaluronic acid isolated after papain digestion of cartilage extracts gave filaments with similar length and size distribution as observed for the central filament both in the intact aggregate and in the trypsin digests. 6. Umbilical-cord hyaluronic acid was also studied and gave electron micrographs similar to those described for hyaluronic acid from cartilage. However, the length of the filament was somewhat shorter. 7. The electron micrographs of both intact and selectively degraded proteoglycans corroborate the current model of cartilage proteoglycan structure.

Proteoglycans are a major component of the intercellular matrix of cartilage. They contain a central protein core (mol.wt. about 200000) to which a large number of negatively charged glycosaminoglycan chains are attached (Hascall & Sajdera, 1970; Heinegård & Hascall, 1974; Heinegård & Axelsson, 1977). The polysaccharides are attached to the polysaccharide attachment region consisting of about two-thirds of the protein. The remaining portion of the core contains few or no polysaccharides and has a structure that allows specific interaction with hyaluronic acid. By such interactions a number of proteoglycan monomers can attach to each hyaluronic acid molecule, forming proteoglycan aggregates (Hascall & Heinegård, 1974*a*; Hardingham & Muir, 1974). The link proteins stabilize the aggregate (Hascall & Heinegård, 1974*b*).

A modified Kleinschmidt technique has been used to examine proteoglycan monomers by electron microscopy, corroborating the current model for proteoglycan structure (Rosenberg *et al.*, 1970; Wellauer *et al.*, 1972; Thyberg *et al.*, 1975). Spread proteoglycan aggregates on cytochrome *c* also give electron-microscopic pictures that would be anticipated from the chemical data (Rosenberg *et al.*, 1975; Kimura *et al.*, 1978).

An important advantage of using electron-microscopic techniques is that parameters of individual molecules can be measured, rather than averages of large numbers of molecules. The present work was undertaken to further establish the structure of cartilage proteoglycans, by using electron-microscopic techniques in combination with selective cleavage of structures in the proteoglycans with specific enzymes. A preliminary account of some of the data presented in this paper has been given previously (Lohmander *et al.*, 1976; Lohmander, 1977).

Experimental

Chemical methods

Bovine nasal cartilage proteoglycan aggregates (A1-fraction§) were prepared by associative CsCl-density-gradient centrifugation of guanidinium

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§ The operational nomenclature suggested by Heinegård (1972) is used throughout this paper. The proteoglycan aggregate preparation is referred to as fraction A1, and the monomer preparation is called fraction A1-D1. Trypsin digestion is indicated by T, and chondroitinase digestion is referred to by CB.

chloride extracts as described elsewhere (Heinegård, 1972). The material in the A1 fraction was recovered as the sodium salt by dialysis of the material against 0.5M-sodium acetate, pH7.0, followed by water and then freeze-drying. A sample of fraction A1 (100mg) was dissolved in 10ml of 0.1M-sodium acetate/0.1M-Tris/HCl buffer, pH7.3, and digested with 0.5 unit of chondroitinase ABC (Miles–Seravac, Stoke Poges, Bucks., U.K.) for 45min at 37°C. These conditions were selected to degrade most of the chondroitin sulphate, while leaving the hyaluronic acid as intact as possible (Heinegård & Hascall, 1974). The digest was chromatographed on a column (2.2cm× 185cm) of Sepharose 2B (Pharmacia Fine Chemicals, Uppsala, Sweden) eluted with 0.5M-sodium acetate, pH7.0. Column effluents were analysed for contents of uronic acids and protein with automated versions of the carbazole and the Folin method respectively (Heinegård, 1973). The void volume peak (fraction A1-CB_{80}) contained the hyaluronic acid with attached proteoglycan protein cores and link proteins, i.e. the aggregated proteoglycans, after the chondroitin sulphate side chains had been removed. The oligosaccharides derived from the chondroitin sulphate chains were eluted in the total volume. Another sample of the A1 preparation was first digested with chondroitinase as described above, and subsequently directly digested with diphenylcarbamoyl chloride-treated trypsin (10μg/mg of A1; Sigma Chemical Co., St. Louis, MO, U.S.A.) as described previously (Heinegård & Hascall, 1974). The digest was chromatographed on Sepharose 2B, and eluted with 0.5M-sodium acetate, pH7.0. The first eluted peak (cf. Fig. 3*a* in Heinegård & Hascall, 1974), which contained the hyaluronic acid–link-protein–hyaluronic acid-binding region complexes, was recovered.

Still another sample of the A1 preparation was digested directly with 10μg of diphenylcarbamoyl chloride-treated trypsin per 5mg of proteoglycan for 3–5h at 37°C. The digest was centrifuged in an associative CsCl density gradient essentially as described elsewhere (Heinegård & Axelsson, 1977). The starting density was 1.65g/ml. The sample (5mg/ml) was centrifuged for 64h at 35000rev./min (95000$g_{av.}$) and 18°C in an MSE 8×25ml angle rotor. The top fraction was recovered and chromatographed on Sepharose 2B. The column (2.5cm×94cm) was eluted with 0.5M-sodium acetate, pH7.0. The first peak, fraction A1-T-A3-2B1, which was only partially included, contained the complex of hyaluronic acid-binding region and link protein. It was recovered, dialysed against water and freeze-dried (Heinegård & Axelsson, 1977).

In another set of experiments a crude hyaluronic acid preparation was isolated from bovine nasal septum. Cartilage was pulverized in liquid N_2 by using a Wiley mill. The powder (30g) was extracted with 10vol. of 4M-guanidinium chloride/0.05M-sodium acetate, pH5.8. After extraction for 24h at 4°C the extract was clarified by centrifugation at 45000g at 4°C for 30min. The extraction residue was washed with 50ml of 4M-guanidinium chloride/0.05M-sodium acetate, pH5.8. After centrifugation as above, the supernatants from the extract were pooled and concentrated by ultrafiltration with an Amicon PM-10 membrane. The retained solutions (about 80ml) were dialysed against water and freeze-dried. The dry weight of the extract was 3.7g. The preparation was dissolved in 250ml of 0.05M-sodium phosphate/0.01M-EDTA/0.05M-cysteine/HCl, pH6.5, and digested with 2×250μl of crystalline papain (Sigma) for 10h, at 37°C, a second lot of enzyme being added after 3h of incubation. Aqueous cetylpyridinium chloride (5%) was added dropwise under continuous stirring to precipitate the glycosaminoglycans from the clear digest (Scott, 1960). The flocculent precipitate obtained, was recovered by centrifugation at 3000$g_{av.}$ for 30min. It was dissolved in 1M-$MgCl_2$, and the molarity of the solution was checked by titration of the chloride ions by using an Aminco chloride titrator. Aqueous cetylpyridinium chloride (0.1%) was then added to bring the concentration to 0.25M-$MgCl_2$, again checked by titration of the chloride ions. The precipitate formed was removed by centrifugation at 3000$g_{av.}$ for 30min. Glycosaminoglycans in the supernatant were recovered as a precipitate after addition of 5vol. of ethanol. The ethanol precipitate was centrifuged at 3000$g_{av.}$ for 30min and the pellet was freeze-dried. The sample was redissolved in 0.02M-Na_2SO_4 and applied to a cetylpyridinium chloride–cellulose column (2cm×30cm) equilibrated with 1% cetylpyridinium chloride as described by Antonopoulos *et al.* (1961). The column was eluted with 4 bed volumes of 1% cetylpyridinium chloride, and the effluent was discarded. Subsequently the columns were eluted with 3 bed volumes of 0.3M-NaCl containing 0.05% cetylpyridinium chloride. The glycosaminoglycans were recovered from the effluent after addition of 5vol. of ethanol, and centrifugation of the precipitate was done as described above. The pellet was washed with ethanol and freeze-dried. Since hyaluronic acid is eluted from a cetylpyridinium chloride–cellulose column with 0.3M-NaCl (Antonopoulos *et al.*, 1961) this material will be referred to as the hyaluronic acid fraction. Its weight was 9.8mg. Hexosamines were separated on an automatic amino acid analyser after hydrolysis of the samples in 4M-HCl under N_2 for 10h at 100°C.

Electron microscopy

Samples were dissolved overnight at 4°C at a concentration of 0.1mg/ml in 0.1M-KCl in 0.05M-potassium phosphate, pH7.0 (Rosenberg *et al.*, 1970).

These stock solutions were stored frozen. Two methods were used for spreading the proteoglycans in a monomolecular layer with cytochrome *c*. The first method was the droplet diffusion technique (Lang & Mitani, 1970) essentially as described by Thyberg *et al.* (1975). The ionic strength of the droplet solution was 0.3M-ammonium acetate, pH5. The second spreading method was the trough technique of Kleinschmidt & Zahn (1959) as modified by Rosenberg *et al.* (1970). A hypophase of 0.3M-ammonium acetate, pH5, was used with a spreading solution of 1M-ammonium acetate, pH5. Irrespective of the spreading method used, proteoglycan/cytochrome *c* films were picked up on copper grids coated with carbon-stabilized Formvar film. The grids were rinsed in 90% ethanol, stained with 0.1mM-uranyl acetate in 90% ethanol for 1 min, rinsed in 90% ethanol for 30s and left to dry. The grids were then rotary-shadowed at an angle of 7° and a distance of 13cm with 10mm of 8mil platinum/palladium (4:1, w/w) wire (Ladd Research Industries, Burlington, VT, U.S.A.). Grids were examined in a Philips EM 300 electron microscope operated at 60kV. Micrographs were made at a magnification of 10000–20000×. Plates were then enlarged about 10 times in a photographic enlarger and the molecules traced on paper. The total magnification was routinely checked with a carbon replica of a ruled diffraction grating having 2160 lines/mm (Ladd Research Industries). Length measurements were made with a micrometer modified into a map ruler with a scale division corresponding to 1mm on the paper.

Molecules to be measured were selected in the following way. Grids were scanned without overlapping. For proteoglycan aggregates, fields containing one or more proteoglycan aggregates with a clearly identifiable central core filament and well extended and separated side chains were photographed. As long as the core filament and the side chains were well extended and discernible no lower limit with regard to size was used. For filamentous molecules, such as hyaluronic acid, all molecules found during scanning of a grid were photographed and measured. No lower limit of size was used.

Results and Discussion

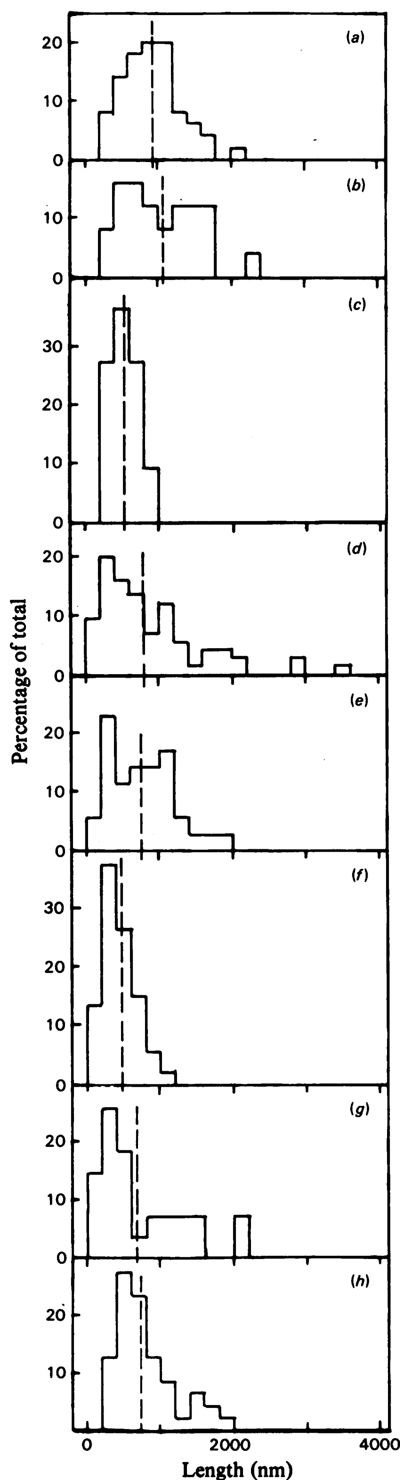
In a previous electron-microscopic study of the structure of proteoglycan monomers, a droplet modification of the Kleinschmidt technique was used (Thyberg *et al.*, 1975). In the present investigation proteoglycan aggregates were studied by using this technique as well as the 'trough technique' introduced by Kleinschmidt & Zahn (1959).

Typical pictures obtained with cartilage proteoglycan aggregates are shown in Plates 1(*a*) and 1(*b*). They are very similar to those previously published by Rosenberg *et al.* (1975). Analogous micrographs were obtained with the trough and the droplet techniques throughout this work. Therefore no special references will be given in the text to the technique used. The aggregates appear as a central filament from which several side-chain filaments radiate out. The current model of the proteoglycan structure is one of a central extended hyaluronic acid chain to which several proteolgycan monomers are attached via one end of the molecule. The monomers are most likely also extended in solution (Pasternak *et al.*, 1973). In the electron micrographs it is likely that the central filament represents the hyaluronic acid and the side-chain filaments the proteoglycan monomers. Physicochemical data indicate that the molecular weight of hyaluronic acid in aggregates from bovine nasal cartilage is in the order of 500000 (Hascall & Heinegård, 1974*a*; Cleland & Sherblom, 1977). The average length of the central filament of the molecules presently studied is 1037nm (Table 1, Fig. 1). Assuming that the hyaluronic acid disaccharides are fully stretched and about 0.96nm long (Atkins *et al.*, 1974) about 1000 would be required to give a glycosaminoglycan chain 1000nm long. The molecular weight of such a polysaccharide would be about 500000. The electron-microscopic data, then, can be used to calculate a molecular weight of the hyaluronic acid in good agreement with values expected from chemical data. The side chains usually appeared as single extended filaments with an average length of 249nm (Table 1, Fig. 2, Plates 1*a* and 1*b*). As in a previous study on proteoglycan monomers (Thyberg *et al.*, 1975), their distribution of lengths did not differ significantly from a normal distribution as estimated by Kolmogorov's test (Keeping, 1962). In some instances the side chains had short extensions, giving them an appearance like that of the proteoglycan monomers observed previously (Rosenberg *et al.*, 1970; Thyberg *et al.*, 1975). It is therefore likely that the side chains represent the proteoglycan monomers, with the chondroitin sulphate chains condensed

Table 1. *Dimension of proteoglycan aggregates in electron micrographs of cytochrome c/proteoglycan monolayers*

The correlation coefficient for length of central filament to number of side chain filaments is 0.92. Values are means±S.D. for the numbers of determinations in parentheses.

Length of central filament (nm)	1037 ±527	(25)
Number of side chain filaments/central filament	29 ± 13.4	
Length of the side chain filaments (nm)	248.9 ± 77.0	(263)
Length of the 'hinge' region (nm)	71.0 ± 18.6	(49)
Average distance between side chain filaments (nm)	35.8	



along the protein core. In separate electron micrographs, proteoglycan monomers appeared as single filaments with no extensions (results not shown). The average length of the side chain is somewhat shorter than that observed for monomers isolated from the same tissue (Fig. 2; cf. Thyberg *et al.*, 1975). It should be pointed out, however, that the somewhat shorter length observed for the monomers in the aggregate electron micrographs is consistent with the current model of proteoglycan structure. The procedure used to isolate proteoglycan monomers (A1-D1) from A1 fractions selects for larger molecules, whereas in the aggregate all sizes of monomers in the extract are represented (Heinegård, 1977). In addition, differences in the technique for spreading monomers and aggregates

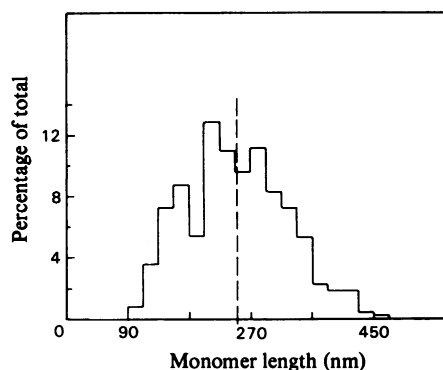
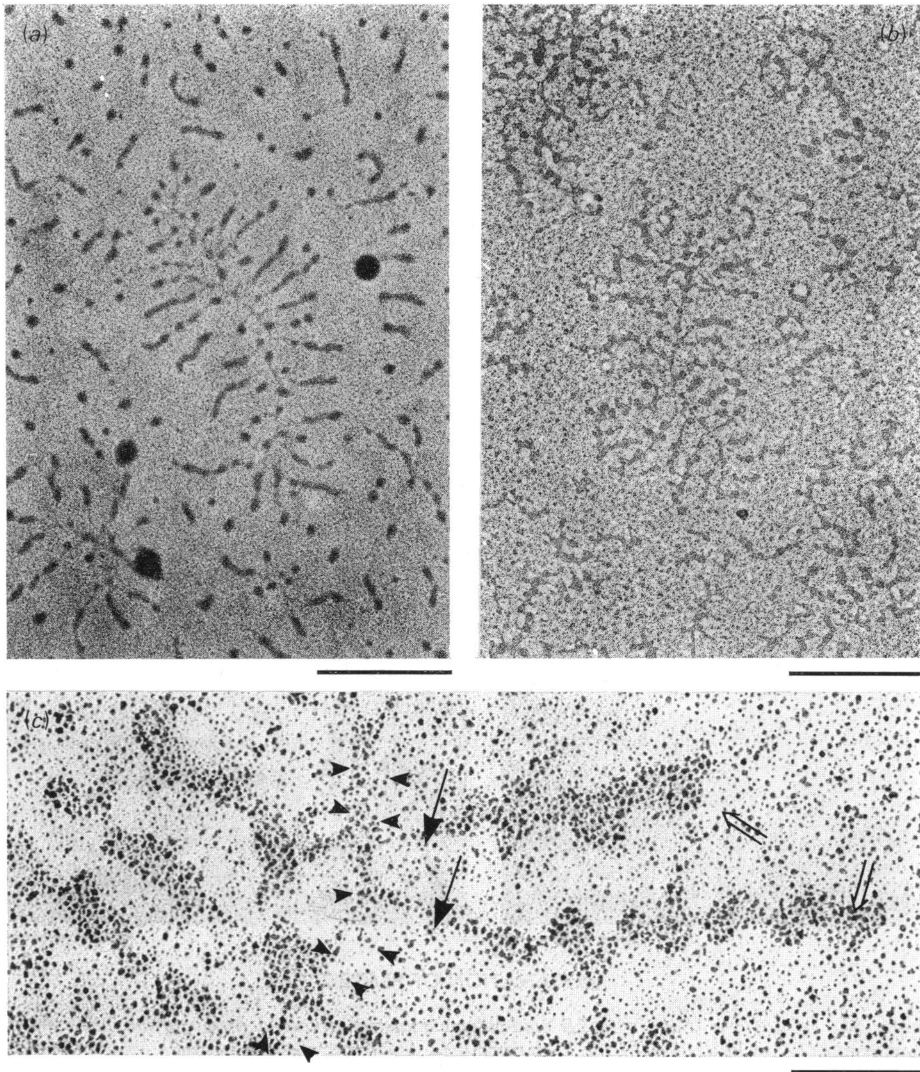


Fig. 2. *Variable length of proteoglycan monomers in proteoglycan A*1 *preparation*
The broken line denotes the mean.

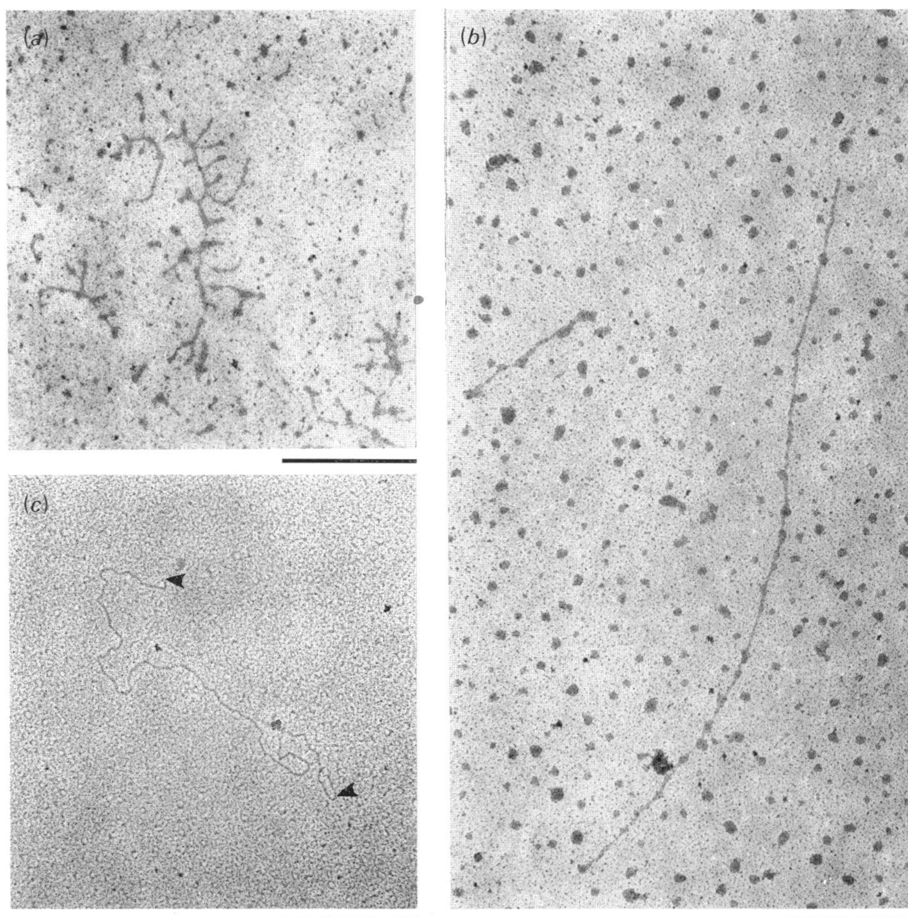
Fig. 1. *Variability of length of proteoglycan monomers in proteoglycan A*1 *preparations*
(*a*) A1 preparation spread with the droplet technique; (*b*) A1 preparation spread with the trough technique; (*c*) spread of the Sepharose 2B-excluded fraction prepared from a limited digest of the A1 preparation (A1-CB_{80}-2B1); (*d*) spread of the large-molecular-size material obtained from a Sepharose 2B chromatogram of the top fraction from a CsCl density gradient of a trypsin-digested A1-preparation (A1-T-A3-2B1); (*e*) spread of cartilage hyaluronic acid prepared by the cetylpyridinium chloride-fractionation procedure; (*f*) spread of the large-molecular-size material prepared by Sepharose 2B chromatography of an A1 preparation that had been digested sequentially to give a partial chondroitinase digest followed by trypsin digestion; (*g*) umbilical-cord hyaluronic acid spread with the trough technique, with a water rinse; (*h*) umbilical-cord hyaluronic acid spread with the trough technique, with the ethanol rinse. The broken line denotes the means.



EXPLANATION OF PLATE I

Electron micrographs of proteoglycan aggregates (*A*1)

(*a*) The specimen was stained with uranyl acetate (bar equals 500nm); (*b*) the specimen was shadowed with platinum/palladium (bar equals 500nm); (*c*) enlarged area of proteoglycan aggregate corresponding to (*b*). ►, central hyaluronic acid filament; ⇒, proteoglycan monomers; →, 'hinge' region of proteoglycan monomer. The specimen was shadowed with platinum/palladium (bar equals 100nm).



EXPLANATION OF PLATE 2

Electron micrographs of (a) the Sepharose 2B void-volume material prepared from partially chondroitinase-digested proteoglycan aggregates (A1-CB_{80}-2B1), (b) the hyaluronic acid, link protein and hyaluronic acid-binding region complex recovered from trypsin-digested proteoglycan aggregates (A1-T-A3-2B1) and (c) hyaluronic acid from umbilical cord

In (*a*) about 80% of the chondroitin sulphate side chains (80% of the uronic acid) had been removed. In (*a*) and (*b*) the specimen was stained with uranyl acetate and (*c*) the material was shadowed with platinum/palladium (bars equal 500nm). ►, End of hyaluronic acid.

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may influence the apparent length of the monomer molecules. The length of the side chain filaments is in good agreement with the values obtained by Rosenberg *et al.* (1975) with bovine articular cartilage proteoglycan aggregates and also with values obtained with proteoglycan aggregates isolated from a transplantable rat chondrosarcoma (G. Hascall, personal communication). As discussed elsewhere, physicochemical data on the length of the protein core correlate well to the data obtained by electron microscopy (Thyberg *et al.*, 1975).

The average distance between the side-chain filaments (35.8nm, Table 1) indicates that about 35–40 hyaluronic acid disaccharides separate the proteoglycan monomers, in good agreement with chemical data, which indicate an approximate distance of 50 hyaluronic acid disaccharides in saturated hyaluronic acid–proteoglycan monomer complexes (Hascall & Heinegård, 1974*a*). The average distance of about 36nm between the monomers in the aggregates that had been dissociated and reassociated is in good agreement with that (33nm) obtained with aggregates isolated from rat chondrosarcoma with a procedure not involving dissociation (G. Hascall, personal communication). These values can be compared with the spacing of 25–30nm for the monomers observed in micrographs of bovine articular cartilage proteoglycan aggregates obtained by Rosenberg *et al.* (1975). Statistical data from the electron micrographs of aggregates indicate that there are an average of 29 side-chain filaments per central filament (Table 1), i.e. proteoglycan monomers per hyaluronic acid molecule. The correlation coefficient (0.92) for length of central filament versus number of side chains indicates that there is a relatively constant number of proteoglycan monomers per unit length hyaluronic acid. If it is assumed that the molecular weight of the monomers is 2×10^6–2.5×10^6 (Pasternak *et al.*, 1973), the average molecular weight of the aggregates measured would be in the order of 6×10^7–7×10^7.

A number of the side chains appear to be connected to the central filament via a hinge region (Plate 1*c*). It is known from chemical data that a peptide region (mol.wt. of peptide about 20000) containing short glycosaminoglycan chains, the keratan sulphate-rich region, is located near the attachment between proteoglycan monomers and hyaluronic acid (Heinegård & Axelsson, 1977). The hinge region may be related to the keratan sulphate-rich region, which if extended should have a length of about 70nm, which is the same as the average length observed for the hinge region (Table 1). In some instances it appears that two side-chain filaments originate from almost the same point at the central filament. It is therefore possible that some of the proteoglycan monomers are clustered along the hyaluronic acid. The exact distance between the monomers along hyaluronic acid was, however, not studied.

The chondroitin sulphate side chains were removed from the proteoglycan monomers in the aggregates by partial chondroitinase digestion and subsequent gel chromatography. The digestion conditions were chosen such as to cleave preferentially the chondroitin sulphate chains and leave the hyaluronic acid as intact as possible (Heinegård & Hascall, 1974). Since the complex of hyaluronic acid, proteoglycan protein cores and link proteins was eluted in the void volume from a Sepharose 2B column, it appeared that the hyaluronic acid remained relatively intact. An extensive degradation of the hyaluronic acid should have liberated single proteoglycan protein cores, which would have chromatographed in the retarded peak of the Sepharose 2B column (Hascall & Heinegård, 1974*a*). The pictures of the complex show the same major features as those of intact aggregates (Plate 2*a*). One point of difference, though, is the shorter central filament in the digested samples [average 541nm; Fig. 1(*c*), A1-CB_{80}-2B1]. It is likely that the chondroitinase digestion had caused an average of one or two breaks in each hyaluronic acid molecule, but too few to decrease the size of the complex such that it would chromatograph in the included volume on Sepharose 2B. With the technique used for spreading aggregates the chondroitin sulphate side chains are folded along the proteoglycan monomer protein core and are therefore not seen. Therefore removal of the chondroitin sulphate chains should be expected not to alter the general appearance of the electron micrographs.

All of the chondroitin sulphate-rich region and the keratan sulphate-rich region were cleaved from the hyaluronic acid-binding region by using trypsin digestion (Heinegård & Hascall, 1974; Heinegård & Axelsson, 1977). The remaining large-molecular-weight complex of hyaluronic acid-binding region–link protein–hyaluronic acid was isolated as described in the Experimental section, and used for spreading with cytochrome *c*. A representative electron micrograph is shown in Plate 2(*b*). The filaments that were observed had an average length of 864nm and showed no side chains. The distribution of the lengths is shown in Fig. 1(*d*) (A1-T-A3-2B1). This value is in good accordance with the length of the central filament observed when the aggregates were used for spreading. It is likely, then, that the filament represents the hyaluronic acid. The proteinaceous hyaluronic acid-binding region and link protein are not seen with the technique. Neither do electron micrographs of proteoglycan monomers contain any structure that could correspond to the hyaluronic acid-binding region (Thyberg *et al.*, 1975). In some electron micrographs, however, globules appeared along the hyaluronic acid at intervals. Possibly the few keratan sulphate chains

known to be present in the hyaluronic acid-binding region (Heinegård & Axelsson, 1977) appeared as globules in some pictures. These structures may represent the hyaluronic acid-binding region still attached to the hyaluronic acid. The trypsin-digestion experiments show that removal of the major portion of the proteoglycan monomers from the hyaluronic acid can be visualized as removal or absence of the side-chain filaments. The observation corroborates the proposed model for proteoglycan aggregate structure (Heinegård & Hascall, 1974; Heinegård & Axelsson, 1977). Electron micrographs similar to those in Plate 2(*b*) were obtained when the corresponding fragment of hyaluronic acid–link protein–hyaluronic acid-binding region complex was prepared from the partial chondroitinase digest of the proteoglycan aggregates. The major difference was that the filament observed had an average length of only 443nm with a distribution of values shown in Fig. 1(*f*) (A1-CB_{80}-T-2B1). Such a result was to be expected, since as discussed above the chondroitinase had probably cleaved a few bonds in the hyaluronic acid.

Hyaluronic acid isolated from cartilage was studied. Cartilage was digested with papain and the hyaluronic acid was purified by precipitation with cetylpyridinium chloride and chromatography on a cetylpyridinium chloride–cellulose column (Antonopoulos *et al.*, 1961). The purified sample contained equimolar amounts of hexosamine and uronic acid, with 80% of the hexosamines being glucosamine. Therefore it contained mainly hyaluronic acid, probably with a small contamination of chondroitin sulphate. The filaments observed by electron microscopy were similar to those observed in a preparation of synovial-fluid hyaluronic acid (Fessler & Fessler, 1966) and were on the average 761 nm long with the variation shown in Fig. 1(*e*) (HA–0.3M-NaCl). These values are somewhat lower than the length of the central hyaluronic acid filament in the aggregates. It is known, however, that in the cetylpyridinium chloride precipitation procedure the glycosaminoglycans are fractionated according to their size (Laurent & Scott, 1964). Elution of hyaluronic acid with 0.3M-NaCl may select for smaller hyaluronic acid molecules. Alternatively some degradation of the hyaluronic acid may have occurred in the isolation procedure. Therefore electron micrographs were made of umbilical-cord hyaluronic acid (a gift from Dr. M. B. Mathews; Plate 2*c*). The observed average length of 774nm (distribution shown in Fig. 1) is in good agreement with the value discussed above for the fraction of cartilage hyaluronic acid. A similar average value (732nm) was obtained when the grids were rinsed with water instead of 90% ethanol after the monolayer was picked up, suggesting that the ethanol rinse did not result in any significant condensation of the molecules. Assuming a disaccharide length of about 0.96nm (Atkins *et al.*, 1974), the length of the filament observed when umbilical-cord hyaluronic acid was spread indicates that the average length was about 800 dissacharides and the mol.wt. about 300000. The data obtained by viscosity measurements indicate a mol.wt. of about 250000 (M. B. Mathews, personal communication), a value close to that observed. The data indicate that a relatively accurate molecular weight for hyaluronic acid can be obtained by using electron microscopy, independent of whether proteoglycan aggregates or isolated hyaluronic acid is used for spreading.

Electron microscopy can be a very useful tool for testing models of the structure of proteoglycans, particularly when combined with specific fragmentation and purification methods. It should be stressed that morphological investigations can only provide supporting evidence for hypothetical models suggested by chemical data. Once the model is established, electron microscopy can be used to collect information about structures of single molecules in polydisperse systems. Such information is more difficult to obtain with chemical or physicochemical methods.

Grants were obtained from the Swedish Medical Research Council (Projects 13X-00139 and 12X-03355), the King Gustaf V 80th Birthday Fund and the Medical Faculty, University of Lund and Karolinska Institutet.

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