Isolation and Characterization of Proteoglycans from Porcine Ovarian Follicular Fluid*

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Monomer proteoglycan was isolated from porcine ovarian follicular fluid by isopycnic CsCl centrifugation in the presence of 4 M guanidine HCl and protease inhibitors. The elution profile of the D1 preparation on Sepharose 2B was similar to that of monomer proteoglycan from bovine nasal cartilage, indicating a similar molecular size. Follicular fluid proteoglycans consist of about 20% protein, 50% dermatan sulfate, and 20% oligosaccharides rich in sialic acid, galactose, mannone, glucosamine, and galactosamine. The amino acid composition of this proteoglycan is significantly different from that of cartilage proteoglycans, with a higher proportion of aspartic acid, threonine, and lysine, and lower amounts of proline and glycine. Alkali-released peptides nearly as small as those released by papain or plasmin were observed on Sephadex G-25, corresponding to pentasaccharide accounts for 9% of total hexuronic acid. Disaccharide units released by chondroitinase ABC consists of 67% 4-sulfated, 22% 6-sulfated, 5% non-sulfated, and 5% disulfated disaccharides. After treatment with 0.05 M NaOH, 1 M NaBH₄ at 45°C for 24 h, two major acid-containing oligosaccharides were observed on Sephadex G-25, corresponding to pentasaccharide and hexasaccharide. The pentasaccharide contained sialic acid, galactose, glucosamine, and galactosamine in the proportions 1:2:1:1. The galactosamine is O-glycosidically linked to the protein core. This oligosaccharide accounts for approximately 77% of all the sialic acid in the follicular fluid proteoglycans. The hexasaccharide fraction contained sialic acid, galactose, mannone, and glucosamine in the proportions 1:2:1:2. It also contained a small amount of fucose and galactosamine. The linkage of these oligosaccharides to the protein core remains to be determined. The follicular fluid proteoglycans, unlike those from cartilage, do not interact with hyaluronic acid. Digestion with trypsin, chymotrypsin, or plasmin released dermatan sulfate peptides nearly as small as those released by papain or alkali; in contrast, cartilage proteoglycans were resistant to plasmin and released peptides containing an average of more than four chondroitin sulfate chains after trypsin or chymotrypsin digestion.

The presence of glycosaminoglycans in ovarian follicular fluid has been previously reported (1-3). These glycosaminoglycans have been partially characterized and their possible physiological roles, particularly with respect to ovulation, were discussed almost two decades ago (3). Jensen et al. (3) prepared glycosaminoglycans from a trypsin-digested follicular fluid by ethanol precipitation of trichloroacetic acid-soluble material and found that glycosaminoglycans constitute 0.2 to 0.3% (w/v) of the follicular fluid. They speculated that chondroitin sulfate and hyaluronic acid were the sources of the galactosamine and glucosamine. They also showed that the galactosamine/glucosamine ratio is smaller in the follicular fluid from small follicles, and that the average molecular weight of glycosaminoglycans from small follicles is higher, 98,000, than that from large follicles, 18,000, as estimated by osmometry. Gebauer et al. (4) recently investigated sulfated glycosaminoglycans in rat ovarian tissues and reported the presence of a heparin-like substance, chondroitin sulfate, and dermatan sulfate. Recent progress in the field of proteoglycan biochemistry has indicated that glycosaminoglycans are not present in tissues as free polysaccharide chains, but are covalently bound to core proteins as part of larger proteoglycan molecules which contain more than one polysaccharide chain. Therefore, the present study was undertaken to determine the characteristics of the native intact proteoglycan molecules to elucidate details about their structure and potential functions in the ovarian follicles.

EXPERIMENTAL PROCEDURES

Materials—Ultrapure guanidine HCl and cesium chloride (biological grade) were purchased from Schwarz/Mann; 6-aminohexanoic acid and benzamidine hydrochloride from Eastman; papain (twice crystallized), diphenylcarbamyl chloride-treated trypsin, plasmin (0.3 unit/mg) and a-chymotrypsin (three times crystallized) from Sigma; chondroitinase ABC (Proteus vulgaris) and chondroitinase AC (Arthrobacter aureus) from Miles; Bio-Gel P-10 from Bio-Rad; Sephadex G-75, Sephadex 25, Sepharose 2B, and Sepharose 6B from Pharmacia; 3% OV-225 on Supelcoport (100/120 mesh) from Supelco Inc.; and Aquasol from New England Nuclear. Hyaluronic acid prepared from umbilical cord was a gift from Dr. Theodore Oegema, University of Minnesota Medical School. "H-labeled hyaluronic acid aggregate) and Al-D1 (monomer), were prepared as described previously (6).†

Collection of Follicular Fluid—Porcine ovaries were obtained at a local slaughterhouse. The ovaries were excised within 20 to 30 min after killing and placed in 0.9% NaCl, 0.1 M 6-aminohexanoic acid, 0.005 M benzamidine hydrochloride, 0.05 M sodium EDTA, and 0.05 M sodium acetate, pH 5.8 at 0°C. All preparative procedures were performed at 0-4°C unless otherwise stated. Follicular contents were

† The nomenclature Al-D1 and D1 define the proteoglycans as described by Heinegdrd (8). The abbreviations used are: BN, bovine nasal cartilage; F₀, ovarian follicular fluid from medium sized follicles; HA, hyaluronic acid; Gal, galactose; NeuNac, N-acetylneuraminic acid; GalNAc, N-acetylgalactosamine; GcNAc, N-acetylglucosamine.
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aspirated from small (1 to 2 mm), medium (3 to 5 mm), or large (6 to 12 mm) ovarian follicles with a syringe and 20-gauge needle within 2 h. The follicular contents were centrifuged at 9000 × g for 30 min to remove cells and debris. The follicular fluid was stored at −20°C until subsequent isolation procedures.

Isolation Procedures—One volume of follicular fluid was mixed with 2 volumes of 6 M guanidine HCl, 0.15 M 6-aminohexanoic acid, 0.0075 M benzamidine hydrochloride, 0.075 M sodium EDTA, and 0.075 M sodium acetate, pH 5.8. Dissociative CsCl density gradients (initial density 1.46 g/ml) were formed in a Beckman SW 50.1 rotor by centrifugation at 37,000 rpm for 48 h, as described previously for cartilage proteoglycans (6, 7). Four equal fractions, designated D1 through D4 from bottom to top, were prepared using a Beckman tube slicer, as described by Heinegard (8) (see Fig. 1 below). The bottom two fractions (D1 and D2) were brought to a density of 1.52 g/ml with solid CsCl and a second dissociative CsCl density gradient was formed in a Beckman SW 50.1 rotor by centrifugation at 37,000 rpm at 18°C for 48 h. Five equal fractions, labeled D1.2-D1.5 through D1.2-D5 from bottom to top, were prepared using a tube slicer.

For associative CsCl density gradients, porcine ovarian follicular fluid was mixed 1:1 with 1 M guanidine HCl, 0.2 M 6-aminohexanoic acid, 0.01 M benzamidine hydrochloride, 0.02 M sodium EDTA, and 0.1 M sodium acetate, pH 7.0. Solid CsCl was added to give an initial density of 1.56 g/ml and the samples centrifuged in a SW 50.1 rotor at 37,000 rpm at 18°C for 48 h. The bottom one-fourth of the tube was collected by a tube slicer and designated as Fraction A1. Fractions from gradients were dialyzed against 0.5 M sodium acetate, pH 7.0, and then water before lyophilization to obtain the proteoglycans as a sodium salt.

Enzymatic Treatments—Samples were digested for 1.5 or 3 h at 37°C with one or more of the following enzymes: chondroitinase ABC (0.025 to 0.25 unit/mg of sample), chondroitinase AC (0.05 or 1 unit/mg of sample), trypsin (10 μg/mg of sample), or chymotrypsin (10 μg/mg of sample), using a 0.1 M Tris, 0.1 M sodium acetate buffer, pH 7.3. Plasmin digestions (100 pg/mg of sample) were done in 0.01 M sodium acetate, pH 7.0 and 0.005 M sodium borohydride, which prevented the degradation of sugar chains by the enzymes. The hydrolysates were dialyzed against 0.5 M sodium phosphate, 0.15 M NaCl, pH 7.4 at 37°C for 1 h. Papain digestion (30 μg/mg of sample) was carried out at 65°C for 4 h in 0.1 M sodium acetate, pH 7.0 containing 0.005 M sodium EDTA and 0.005 M cysteine hydrochloride.

Alkaline Borohydride Treatment—Treatment with alkaline borohydride was done in 0.05 M NaOH at 45°C for 24 h with 1 M sodium borohydride, which prevented the degradation of sugar chains by the "peeling" reaction (9). Excess borohydride was destroyed by neutralization of the solution with glacial acetic acid. The concentrations of samples were 1 to 5 mg/ml in buffer in all cases. Enzymatic digest or alkaline borohydride-treated samples were chromatographed immediately or after storage at −20°C.

Analytical Procedures—Analyses for total hexosamines were done by the Elson-Morgan reaction (10), while separate hexosamine and hexosaminitol analyses were done with a Durrum amino acid analyzer (11). The iduronic/glucuronic acid ratios were determined by the orcinol-carbazole methods (13-15). Sialic acid was determined by the procedure of Elson-Morgan reaction (17).

Analytical Sepharose 2B, Sepharose 6B, Sephadex G-75, and Sephadex G-25 gel filtration columns (110 X 0.7 cm) were prepared and used to determine the relative molar masses of the samples. The combined D1-D2 fraction was further analyzed by a gel P-10 column, eluted with 0.1 M pyridinium acetate buffer, pH 5.0.

Neutral sugars were determined as glycitol acetate derivatives by gas-liquid chromatography (18). Samples (1 mg) were hydrolyzed in sealed tubes under N2 in 6 M HCl at 110°C for 24 h. The hydrolysates were passed through Dowex 50-X2 (H+ form, 200/400 mesh) and Dowex 1-X8 (acetate form, 200/400 mesh) and washed with water. The effluents were lyophilized and reduced with 200 μl of 0.2 M NaBH4 in 0.01 M NaOH for 1 h at room temperature. The reaction was stopped by the addition of 100 μl of glacial acetic acid. Borate was removed as the volatile trimethyl borate by adding 200 μl of a 1:1,000 v/v concentrated HCl/methanol solution followed by concentration to dryness in a rotary evaporator, repeated three times. The samples were acetylated with 500 μl of an acetic anhydride/pyridine mixture (1:1 v/v) at 100°C for 30 min and dried. The residues were dissolved in 5 μl of pyridine and 0.5-μl aliquots were analyzed. The neutral sugar derivatives were separated with a Hewlett-Packard model 5790 gas-liquid chromatograph equipped with a flame ionization detector. The glass column (0.2 × 180 cm) was packed with 3% OV-225 on Supelcoport. The column was run under isothermal conditions at 180°C, injector temperature 250°C, detector 250°C, N2 15 ml/min, H2 20 ml/min, and air 150 ml/min. Peak areas were used to determine concentrations.

Radioactivity in the effluent fractions from column (0.1 ml sample, pH 10 ml of Aquasol with 5% water) was determined with a Beckman LS 2800 scintillation counter without correction for quenching. The relative contents of unsulfated and of 4- and 6-sulfated disaccharides were estimated by the procedures of Saito et al. (19). Analitical Sepharose 2B, Sepharose 6B, Sephadex G-75, and Sephadex G-25 gel filtration columns (110 X 0.7 cm) were prepared and eluted with 0.5 M sodium acetate, pH 7.0 (20). A Bio-Gel P-10 column (110 X 0.7 cm) was eluted with 0.1 M pyridine acetate buffer, pH 5.0 (21).

Isolation and Characterization of Protein Core Preparation and Oligosaccharides Preparation—The D1 preparation was dissolved (approximately 3 mg/ml) in 0.1 M Tris, 0.1 M sodium acetate, pH 7.3, then digested with chondroitinase ABC (0.05 unit/ml) at 37°C for 1.5 h. The digest was chromatographed on a Sepharose 6B column (110 X 0.7 cm) eluted with 1 M NaCl, 0.5 M sodium acetate (0.1 M sodium sulfate, pH 7.0). Fractions of 0.5 ml were collected and measured for UV absorbance at 280 nm. The fractions corresponding to the protein core peak were collected (Fig. 7B), dialyzed against water, and lyophilized. The yield of material from this peak, subsequently referred to as the protein core preparation, was about 0.75 mg. The hexosamines, hexosaminitol, and hexuronic acid contents of this preparation were measured. For preparation of oligosaccharides, the D1 preparation was treated with alkaline borohydride, chromatographed on a Bio-Gel P-10 column, eluted with 0.1 M pyridinium acetate buffer, pH 5.0. Hexoses and sialic acid contents were measured on each 0.6-ml fraction. The hexose- and sialic acid-containing peaks were collected and the samples lyophilized directly (Fig. 9B). Sialic acid, neutral sugars, hexosamines, and hexosaminol analyses were done as described above.

Hyaluronic Acid Assay and the Interaction of the Follicular Fluid D1 Fraction with Hyaluronic Acid—The amounts of hyaluronic acid in samples in standard were determined by the procedures of Hardingham and Adams (22). Follicular fluid D1 through D4 samples (100 μg) were digested with papain for 4 h. After heat inactivating the papain (100°C, 5 min), samples to be tested were incubated with 600 pg of bovine nasal cartilage D1 preparation at 4°C for 24 h in 0.5 M sodium acetate, 0.1 M sodium sulfate buffer, pH 7.0, and then chromatographed on an analytical Sepharose 2B column with the same buffer. The eluted fractions were analyzed for hexuronic acid and the ratio of complexed proteoglycans to monomer proteoglycans was estimated by calculating peak areas corresponding to the column void volume (aggregate) and to the included peak (monomer). Varying amounts of standard hyaluronic acid samples (from umbilical cord) were analyzed under identical conditions to construct a standard calibration curve. As little as 0.1 μg of standard hyaluronic acid could be detected by this procedure.

A sample of the follicular fluid D1 preparation, 200 μg in 200 μl, was mixed with 1/5 (w/w) of hyaluronic acid in a 0.5 M sodium acetate, 0.1 M sodium sulfate buffer, pH 7.0. After 24 h at 4°C the mixture was chromatographed on Sepharose 2B and analyzed by the same chromatographic method as the hyaluronic acid assay to determine whether follicular fluid D1 molecules could interact with hyaluronic acid.

RESULTS

Isolation of Follicular Fluid Proteoglycan—Proteoglycans were isolated from porcine ovarian follicular fluid by CsCl equilibrium density gradient centrifugation under dissipative conditions to remove noncovalently bound proteins. Preliminary extraction procedures were not necessary, since the proteoglycans were already present in a fluid phase. After the first density gradient centrifugation, approximately 39% and 29% of the total hexuronic acid was recovered in the D1 and D2 fractions, respectively (Table I). These losses may be an underestimate because of possible interference in the carbohydrate reaction by contaminants in the D3 and D4 fractions. The combined D1-D2 fraction was further analyzed by a...
second CsCl equilibrium density gradient centrifugation, again under dissociative conditions but with a higher initial density (Fig. 1). The results of hexuronic acid, protein, and sialic acid measurements, amino acid analyses, and gel filtration on a Sepharose 2B column for each fraction are shown in Tables I to III and Fig. 2. The hexuronic acid/protein ratio rapidly increases with the buoyant density of the material. The sialic acid/protein ratio also increases with the buoyant density of the material, but to a lesser extent than for hexuronic acid; and the sialic acid/protein ratio shows little change in D1,2-D3 fractions. Gel filtration profiles show the presence of one major molecular species, containing hexuronic acid, protein, and sialic acid, that eluted as a broad peak, with $K_v$ of 0.26 to 0.32. The $K_v$ of these peaks decrease with increasing density, suggesting that higher density fractions contain molecules of larger hydrodynamic size. This material accounts for approximately 95% of the total hexuronic acid recovered in the D1 and D2 fraction of the first density gradient. Little or no difference was seen in the amino acid compositions of the bottom three fractions, D1,2-D3 through D1,2-D3, indicating that the protein in these fractions is similar, although the proportion of carbohydrate varies. This major molecular species markedly decreases in concentration in the upper two fractions, while another hexuronic acid-containing component, which is smaller in molecular size, appears in the D1,2-D3 fraction. These findings suggest that the hexuronic acid-containing material recovered in the D3 and D4 fractions of the first density gradient (which accounts for up to 38% of the total hexuronic acid in the follicular fluid) differs from the major molecular species recovered from Fractions D1 and D2. The low buoyant density, smaller component was not studied further.

Fig. 3 shows the elution profile of hexuronic acid from

![TABLE I](image)

| Fraction | Density (g/ml) | Protein (mg/ml) | Hexuronic acid (as glucuronic acid) (µg/ml) | Sialic acid (µg/ml) |
|----------|---------------|----------------|--------------------------------------------|---------------------|
| D4       | 1.35          | 15.5           | 88*                                        | 5.7                 |
| D3       | 1.39          | 2.3            | 58*                                        | 25                  |
| D2       | 1.46          | 0.22           | 85                                         | 386                 |
| D1       | 1.55          | 0.19           | 150                                        | 790                 |

* Overestimate due to nonspecific color reaction from high protein concentration.

Ovarian Follicular Fluid

Mixed with 2 volumes of 6M guanidine HCl and protease inhibitors

Dissociative CsCl gradients in 4M guanidine HCl pH 5.8

initial $\rho = 1.46$

Beckman SW50.1 Rotor

37,000 rpm, 18 h, 48 hr.

Dissociative CsCl gradients in 4M guanidine HCl pH 5.8

initial $\rho = 1.52$

Beckman SW50.1 Rotor

37,000 rpm, 18 h, 48 hr.

Fig. 1. Preparation and fractionation of proteoglycans from porcine ovarian follicular fluid
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The eluent was analyzed for absorbance at 260 nm, hexuronic acid, sialic acid, and protein. The elution profile suggests that the follicular fluid proteoglycans are polydisperse, with a $K_{av}$ of 0.27, which is very close to that for cartilage proteoglycans (Fig. 3). Thus, the average molecular size of the follicular fluid proteoglycan is similar to that of hyaline cartilage proteoglycans. An absorbance spectrum of the D1 preparation showed a higher extinction at 260 nm than at 280 nm, suggesting the presence of some nucleic acid. The D1 preparation was chromatographed on Sepharose 6B and UV absorption was measured for each fraction at 260 nm (Fig. 4). The void volume ($V_v$) peak contained hexuronic acid, protein, and sialic acid, while the peak at the column total volume ($V_t$) did not. The chemical composition of the material at the peak in the void volume was essentially the same as that of the original D1 preparation, except for the absence of ribose in a neutral sugar analysis, further suggesting that the D1 sample contained some RNA (nucleic acid) contamination. The amount was estimated as less than 5% of the dry weight of the D1 preparation from the absorbance spectrum and the known molar extinction coefficient of RNA (25). Small amounts of nucleic acid have been observed in D1 preparations isolated from aortic tissue by procedures similar to those used in this study (26, 27).

### Table IV

| Composition of the porcine ovarian follicular fluid proteoglycan (D1) | Protein core preparation |
|---------------------------------------------------------------|--------------------------|
| **mg/g dry wt** | **μmol/g dry wt** |
| Protein (Lowry) | 200 | 340 |
| Sum of amino acid residues | 175 |
| Glucosamine | 1130 | 223 |
| Glucosamine | 170 | 249 |
| Sialic acid | 192 | 322 |
| Hexuronic acid | 981 | — |
| Glucuronic acid | 894 |
| Iduronic acid | 87 |
| Galactose | 272 | 532 |
| Fucose | 8 | 12 |
| Xylose | 9 | 24 |
| Mannose | 41 | 61 |
| Glucose | 44 | 61 |

Nucleic acid component was removed by Sepharose 6B chromatography. Calculated using both orcinol and carbazole methods. Nonspecific color due to protein or other substances.

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Sepharose 2B chromatography for a D1 fraction isolated from medium-sized porcine ovarian follicles and that for a bovine nasal D1 preparation. The D1 fractions from small and large follicles showed essentially the same profiles as did the D1 preparation from medium-sized follicles. Further, the A1 preparation from medium-sized follicles showed the same profile as did the D1 preparation; there was no evidence of aggregate formation as is observed for cartilage proteoglycans (23, 24). The elution profile suggests that the D1 sample contained some RNA (nucleic acid) contamination. The amount was estimated as less than 5% of the dry weight of the D1 preparation from the absorbance spectrum and the known molar extinction coefficient of RNA (25). Small amounts of nucleic acid have been observed in D1 preparations isolated from aortic tissue by procedures similar to those used in this study (26, 27).

Composition of Follicular Fluid Proteoglycan—The chemical composition of the follicular fluid D1 fraction after Sepharose 6B chromatography to remove nucleic acid contamination is summarized in Table IV. The protein content is higher than that for proteoglycans from bovine nasal cartilage, and accounts for about 20% of the dry weight of the preparation. The amino acid composition also differs significantly from that reported for monomer proteoglycans isolated from cartilage (28): the follicular fluid proteoglycans contain more aspartic acid, threonine, and lysine, and less proline and glycine. Sepharose 6B chromatography of a D1 preparation after digestion with chondroitinase ABC indicated that essentially all the hexuronic acid was released from the core protein (Fig. 5D). The presence of hexuronic acid and galactosamine in approximately equimolar concentrations also indicates the presence of chondroitin sulfate or dermatan sulfate. A comparison of the carbazole/orcinol ratio (13-15), suggests that about 9% of the hexuronic acid is iduronic acid. After chondroitinase AC digestion under conditions which are known to degrade chondroitin sulfate almost completely (0.05 unit/mg of sample, 37°C, 1.5 h), followed by papain digestion to release the undigested dermatan sulfate chains from core protein (29), only 23% of the total hexuronic acid eluted at the column total volume ($V_t$) on a Sepharose 6B column (Fig. 5B). Even after digestion with excess chondroitinase AC (1 unit/mg of sample, 37°C, 3 h), followed by papain digestion, only 50% of the total hexuronic acid eluted at $V_t$ (Fig. 5C). The carbazole/orcinol ratio across the first peak ranges from 0.7 to 0.9, which

The term dermatan sulfate is used to indicate polysaccharide chains which contain both iduronic and glucuronic acid residues and not to indicate only those disaccharides in such chains which contain iduronic acid.
indicates that the iduronic acid/glucuronic acid ratio is higher than in the intact chains, but these partially digested fragments still contain a high proportion of glucuronic acid, consistent with the fact that only about 9% of the total is iduronic acid. This is probably due to the fact that chondroitinase AC from *Arthrobacter aurescens* exhibits primarily exoenzyme activity and very little endoenzyme activity (30). Both papain and alkaline borohydride treatment gave essentially identical hexuronic acid elution profiles on Sepharose 6B. The fractions were analyzed for hexuronic acid by both the carbazole and orcinol procedures. The ratio was constant across the peak (average 0.89) within experimental error, indicating that the proportion of iduronic acid to glucuronic acid in the chains was independent of chain size (Fig. 5A). The chondroitinase AC-resistant fragments, indicative of the presence of iduronic acid, are smaller than undigested chains which can be released by papain or alkali treatment (29). This suggests that the dermatan sulfate is a co-polymer with iduronic acid and glucuronic acid residues in the same chain, as in the case of aortic proteoglycans, knee joint cartilage, umbilical cord, and skin dermatan sulfate (31–36). The average molecular size of the dermatan sulfate chains in the D1 preparation was determined by chromatography of papain- or alkali-treated D1 samples on Sepharose 6B (Fig. 5A). The hexuronic acid eluted from the column as a rather broad peak, with an average $K_{av}$ of 0.30. This $K_{av}$ would correspond to an apparent molecular weight of 56,000, provided the Sepharose 6B used in these

![Figure 5](image)

**Fig. 5.** Sepharose 6B elution profiles of follicular fluid proteoglycan (D1) after treatment with A, papain; B, chondroitinase AC (0.05 unit/mg of sample, 37°C, 1.5 h) followed by papain; C, chondroitinase AC (1 unit/mg sample, 37°C, 3 h) followed by papain; and D, chondroitinase ABC (0.05 unit/mg of sample, 37°C, 1.5 h). The eluents were analyzed for hexuronic acid. The inset C/O is the carbazole/orcinol ratio ($\Delta$).

![Figure 6](image)

**Fig. 6.** Paper chromatography of chondroitinase ABC-treated follicular fluid proteoglycan (D1). The chromatograph was run for 18 h in 1-butanol, acetic acid, 1 M ammonia (2:3:1, v/v). Paper was dried, cut into 1-cm strips, and extracted with water. The absorbance was measured at 232 nm. An unlabeled arrow between origin and 6-sulfated isomer position indicates unidentified peak, presumably disulfated disaccharide. $\Delta$Di-6S, 6-sulfated unsaturated disaccharides; $\Delta$Di-4S, 4-sulfated unsaturated disaccharides; $\Delta$Di-0S, nonsulfated unsaturated disaccharides.

![Figure 7](image)

**Fig. 7.** Gel filtration on A, Sepharose 2B; and B, Sepharose 6B of follicular fluid proteoglycan (D1) after treatment with chondroitinase ABC. The eluents were analyzed for sialic acid, hexuronic acid, and protein. The arrow near $V_i$ shows the elution position for protein components in the chondroitinase ABC preparation alone. Shaded bar designates fractions which were pooled for further study as protein core preparation. The protein and sialic acid peak at $V_i$ is due to nonspecific color reactions by disaccharides and Tris.
experiments had the same elution properties as that used by Wasteson (37). This estimate agrees well with the ratio of xylose to hexuronic acid in the D1 samples, approximately 1:110 (Table IV), which indicates that the average number of the repeating disaccharide units per chain would be about 110, corresponding to a molecular weight of about 51,000. Based on the hexuronic acid content of the D1 preparation, the sodium salt of the dermatan sulfate should be approximately 50% of the dry weight of the sample.

Paper chromatography was used to separate the unsaturated disaccharides generated by digestion of the D1 preparation with chondroitinase ABC. The results (Fig. 6) indicate that 67% of the disaccharides were 4-sulfated, 22% 6-sulfated, and 5% nonsulfated. A small peak between the origin and the 6-sulfated disaccharide suggests the presence of disulfated disaccharides (19), which account for about 5% of the total, and probably contain iduronic acid (38). However, which fractions contain iduronic acid remains to be determined. The glucose present in the D1 preparation might have been derived from small amounts of glycogen, which have been shown to be present in human ovarian follicles by histochemical techniques (39).

Characterization of Protein Core Preparation and Sialic Acid-containing Oligosaccharides—The follicular fluid D1 preparation contained approximately 6% sialic acid by weight. On Sepharose 2B chromatography of the D1 preparation, the elution profile of sialic acid was essentially identical with those of hexuronic acid and protein (Fig. 3). Further, the chromatogram for the D1 preparation after chondroitinase ABC digestion shows that the sialic acid co-elutes on Sepharose 2B with protein (Kav = 0.52), except for the small protein peak which comes from the chondroitinase ABC preparation (Fig. 7A). These data indicate that the sialic acid-containing material is associated with the proteoglycan molecules and is resistant to chondroitinase ABC digestion. A protein core preparation was isolated after removing the dermatan sulfate chains with chondroitinase ABC by Sepharose 6B chromatography (Fig. 7B). The peak material was isolated and its composition determined. The ratios of protein to sialic acid, glucosamine, galactose, and xylose did not change significantly from the original D1 preparation (Table IV). Alkaline borohydride treatment of this protein core preparation (9) converted ap-
proximately 85% of the galactosamine residues into galac-
tosaminol residues; the galactosamine/glucosamine ratio
changed from 0.89 to 0.13 by alkaline borohydride treat-
ment, suggesting that these galactosamine residues were attached to
the protein core of the proteoglycan by O-glycosidic bonds.
The remaining nonreduced galactosamine residues probably are
derived from residual repeat disaccharides near the linkage
regions of the dermatan sulfate chains, which resist chondro-
tinase ABC digestion (40).

D1 samples were treated with alkaline borohydride, chromo-
atographed on Sephadex G-75, and the elution profiles of
hexuronic acid and sialic acid determined (Fig. 8A). ESSentially all
the hexuronic acid eluted in the void volume, and all
the sialic acid-containing materials were released by this treat-
ment (Kav = 0.92). D1 samples were also treated with papain,
chromatographed on Sephadex G-75, and the elution profiles
for hexuronic acid and sialic acid were determined (Fig. 8B).
Again, the uronic acid-containing peak was excluded by this
column, while the sialic acid eluted as a very broad included
peak than after alkaline borohydride treatment, this indi-
cating approximately 85% of the galactosamine residues into galac-
tosamine/glucosamine ratio suggesting that these galactosamine residues were attached to
the protein core of the proteoglycan by O-glycosidic bonds.

The major sialic acid-containing oligosaccharide was determined by Sephadex G-25
chromatography after alkaline borohydride treatment of the
D1 preparation. Two major peaks were observed (Fig. 9A),
and the molecular sizes of these oligosaccharides were esti-
mated by co-chromatography with 3H-labeled oligosac-
charides of hyaluronic acid. A major sialic acid-containing
peak eluted between tetra (HA1)- and hexa (HA6)-saccharides,
and a minor sialic acid-containing peak eluted between HA6
and HA8. Sufficient amounts of these oligosaccharides for the
chemical analyses were isolated from an alkaline borohydride-
treated D1 preparation by chromatography on a Bio-Gel P-10
column (0.7 X 110 cm), eluted with 0.1
buffer, pH 5.0. Peak fractions were collected and lyophilized
directly (Fig. 9B). The results of sialic acid, hexosamine,
hexosaminitol, and neutral sugar analyses are shown in Table
V. The major sialic acid-containing oligosaccharide, eluting as
a pentasaccharide, accounted for 77% of all the sialic acid. It
contained sialic acid, galactose, galactosaminitol, and gluco-
samine in the proportions 1:2:1:1. The data suggest that this
oligosaccharide is a pentasaccharide containing a hex-
saccharide fraction remains to be determined.

No hexosaminitol was
detected. Small amounts of fucose and galactosamine were
present. This oligosaccharide fraction, then, could contain
small amounts of differing oligosaccharides or perhaps some
contamination from the dermatan sulfate. The linkage region
of the oligosaccharides to the core protein for the hexasac-
charide fraction remains to be determined.

Susceptibility to Enzymatic Digestion—Samples of the fol-
llicular fluid D1 preparation were treated with various enzymes
or alkaline borohydride, and the resulting solutions analyzed by
Sepharose 2B chromatography. Essentially the same hex-
uronic acid elution profile was obtained after papain digestion
and treatment with alkali, conditions which are known to
release single chondroitin sulfate chains from cartilage proteo-
glycan (29). The relatively broad peak centered at Kav = 0.71
indicates the polydispersity of dermatan sulfate chains (Fig.
10A). The hexuronic acid elution profiles observed after either
trypsin, chymotrypsin, or plasmin digestion were essentially
identical, with a Kav of about 0.67 (Fig. 10, B to D). The
dermatan sulfate-peptide fragments released by these en-
zymes are therefore only slightly larger than those released
by papain. These results suggest that the individual dermatan
sulfate chains of follicular fluid proteoglycan are separated from each other by peptide regions which are suscepible
to these various specific proteases. In contrast, limit digestion of
bovine nasal proteoglycans with trypsin plus chymotrypsin
yields peptides with an average of four chondroitin sulfate

![Fig. 10. Gel filtration profiles on Sepharose 2B of follicular fluid
proteoglycan (D1) after treatment with various proteolytic enzymes.
A, papain; or alkaline borohydride treatment; B, trypsin; C, chymo-
trypsin; D, plasmin. A gel filtration profile of bovine nasal cartilage
proteoglycan A1 preparation after plasmin treatment with identical conditions is
superimposed (- - -). The eluents were analyzed for hexuronic acid.
](image-url)
Proteoglycans from Porcine Ovarian Follicular Fluid

The data presented here suggest that the proteoglycans isolated from porcine ovarian follicles differ in many respects from the well-characterized proteoglycans from cartilage, although both are of similar average hydrodynamic size. The follicular fluid proteoglycans contain dermatan sulfate chains, which have an average molecular weight of 56,000, which is more than twice as large as that of chondroitin sulfate chains from cartilage proteoglycans ($M_f \approx 25,000$) (42), and are larger than most chondroitin or dermatan sulfate chains reported to date (43). The protein core of the follicular fluid proteoglycans after chondroitinase ABC digestion appears to be larger (44, 45), has a different amino acid composition, and most interestingly, exhibits a different susceptibility to enzymatic digestion than the corresponding core protein derived from cartilage proteoglycans. The follicular fluid proteoglycans do not interact specifically with hyaluronic acid to form the high molecular weight complexes which are responsible for aggregate formation by cartilage proteoglycans. Approximately 20% of the dry weight of the follicular fluid proteoglycan consists of two major oligosaccharides which contain sialic acid. There have been no previous reports that either of these oligosaccharides are present in cartilage or other proteoglycans. Skeletal cartilage proteoglycans, which have an average molecular weight of about 400,000 with an average of about 20 dermatan sulfate chains and 350 sialic acid-containing oligosaccharides attached.

The composition of the major oligosaccharide component (pentasaccharide) of the follicular fluid proteoglycan resembles that of some of the oligosaccharides found in various glycoproteins, such as the blood group-specific glycoprotein isolated from human or porcine ovarian cyst fluid, gastric, and submaxillary mucins (47). The oligosaccharides found in these glycoproteins are very heterogeneous, consist mainly of variable amounts of N-acetylgalactosamine, N-acetylgalactosamine, galactose, and fucose, have N-acetylgalactosamine as a linkage sugar to hydroxyl groups of serine or threonine and show blood group specificities by the substitution of "precursor" or core oligosaccharides with the specific sugar residues (48). One of the precursor tetrasaccharides found in a blood group-specific glycoprotein from human ovarian cyst fluid, namely $\alpha$-Gal$\alpha_1 \rightarrow 3 [\beta$-Gal$\alpha_1 \rightarrow 4 \beta$-GlcNac$\beta_1 \rightarrow 6] \beta$-GalNAc$\beta_1$ (49), has the same composition as the pentasaccharide of follicular fluid proteoglycan except for the absence of a sialic acid residue. Both the blood group-specific glycoprotein and the proteoglycan are derived from ovarian tissue. The glycoprotein has been found in pathological ovarian cyst fluid, but not in normal follicles. These two materials have similar oligosaccharide composition, but differ in many respects (50). The blood group-specific glycoproteins have more highly heterogeneous oligosaccharides as their main component, do not contain glycosaminoglycans, and have a different amino acid composition. A recent report on the characterization of the blood group-specific glycoprotein from human ovarian cyst fluid by Bhaskar et al. (50) suggests that cyst fluid contains little or no proteoglycan of the type described in this paper.

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**Fig. 11.** Gel filtration on Sepharose 2B of A, follicular fluid proteoglycan (D1); and B, bovine nasal cartilage proteoglycan (D1), before (---) and after (---) incubation with 1% (w/w) of umbilical cord hyaluronic acid. The eluents were analyzed for hexuronic acid. The hexuronic acid elution profile was essentially identical with hyaluronic acid and then chromatographed on Sepharose 2B. The hexuronic acid elution profile was essentially identical with hyaluronic acid and then chromatographed on Sepharose 2B. The hexuronic acid elution profile was essentially identical with hyaluronic acid and then chromatographed on Sepharose 2B.
Proteoglycans from Porcine Ovarian Follicular Fluid

One of the oligosaccharides of the epiglycanin, a membrane glycoprotein found in TA3-Ha murine mammary carcinoma ascites cells by Codington et al. (51), has the same composition and linkage sugar galactosamine as the pentasaccharide of follicular fluid proteoglycan.

The physiological roles of the proteoglycans in the ovarian follicles are still unknown. However, on the basis of their molecular characteristics, one can infer that the high viscosity of follicular fluid can be attributed in large part to the proteoglycans (52). This viscosity might help to provide an appropriate spatial and physical environment for granulosa cells or oocytes. The ability of intact proteoglycans to retain solvent in their structure should maintain the follicle in a distended state, and the production of proteoglycans and their secretion into follicular space is likely to contribute to antrum development and follicular growth.

Zachariae (53) demonstrated by autoradiography that [35S]sulfated molecules were secreted into follicular fluid at the time of antrum formation in the rabbit ovary. Thorsoe (54) showed that the [35S]sulfate was incorporated into glycosaminoglycan chains we estimated that the maximum osmotic pressure based on the chemical data described above would be 0.015 mOsm. Strickland et al. (56) have recently demonstrated that plasminogen activator activity in ovarian follicular fluid rises at the time of ovulation, and suggested that the plasmin generated by the plasminogen activator might decrease the tensile strength of the follicular wall and contribute to follicular rupture. Since plasmin was very effective in degrading the follicular fluid proteoglycan as described above, the rise in plasmin activity at the time of ovulation might also reduce the viscosity of follicular fluid by cleaving the protein core of the proteoglycans and thereby facilitate the escape of the oocyte from the ruptured follicle. These potential physiological roles for ovarian follicular fluid proteoglycans remain to be investigated.

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