Isolation and Characterization of Proteochondroitin Sulfate from Pig Skin*

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Proteoglycans were extracted from pig skin with 4 M guanidine HCl at 4°C in the presence of protease inhibitors and were precipitated with ethanolic KSCN to separate them from collagen. They were fractionated into two major components on the basis of buoyant density in a CsCl gradient under associative conditions. Proteodermatan sulfate was present in the lighter fractions, and it contained 50 to 60% protein.

The densest fraction contained proteochondroitin sulfate, which was further purified in a dissociative (4 M guanidine HCl) density gradient. It had a protein content of 4 to 5%, like the proteoglycan of hyaline cartilage, but was smaller as judged by chromatography on Sepharose 2B. No difference in size was seen between proteochondroitin sulfate prepared in associative or dissociative gradients, nor was there any binding to hyaluronic acid. A control experiment showed that proteoglycan of bovine nasal septum, isolated in the presence of skin under identical conditions, was able to bind normally to hyaluronic acid. As determined by cellulose-acetate electrophoresis and enzymatic digestibility, the glycosaminoglycan component was almost entirely chondroitin sulfate (85% 4-sulfate and 15% 6-sulfate), and gel chromatography indicated a M, of 20,000. About 8% of the hexosamine was glucosamine; on the basis of enzymatic susceptibility this was not hyaluronic acid, but might be heparan sulfate and keratan sulfate or keratan sulfate-like oligosaccharides. Amino acid composition of the proteochondroitin sulfate was similar to that of cartilage proteoglycan, with large amounts of serine, glutamic acid, and glycine; cystine and methionine were not detectable. β Elimination caused the destruction of 4 times as much serine as threonine, but the linkage sites of chondroitin sulfate cannot be specified.

Proteoglycans and collagen are important structural macromolecules in the intercellular spaces of most connective tissues. The PG contribute to the mechanical properties of the tissues and may also be involved in the formation and organization of collagen fibrils (2, 3). PG consist of glycosaminoglycans chains covalently attached to protein. Those extracted from bovine nasal cartilage, which contain both chondroitin sulfate and keratan sulfate, have been purified and extensively characterized (see review by Hascall (4)). They are polydisperse and have average molecular weights of a few million, and most of the molecules exist in the form of very large aggregates as a result of their ability to bind specifically to hyaluronic acid, both in the tissue (5) and after isolation. PG of tissues that contain predominantly dermatan sulfate, such as dermis, aorta, and heart valves, have been less thoroughly studied, partly because of the greater difficulty of extraction. In aorta, there is evidence for a PG in which dermatan sulfate and chondroitin sulfate are attached to the same protein core (6, 7), and another component of such tissues, heparan sulfate, also appears to be in PG form.

Toole and Lother (8) extracted 88% of the dermatan sulfate from bovine skin with 6 M urea at 60°C for 16 h, but they did not purify and characterize the PG further. Obrink (9) extracted pig skin for four 24-h periods in 6 M urea at 60°C. Following isopycnic centrifugation, the PG contained 92% of its hexosamine as dermatan sulfate with trace amounts of chondroitin sulfate and heparan sulfate. The protein content was 58%, and the preparation was polydisperse, the major portion having an average molecular weight of 2.9 x 10^6. The extraction procedures used by these two laboratories might have caused structural alterations in complex macromolecules, and milder procedures would be desirable. This report describes the extraction of PG from pig skin with 4 M guanidine HCl at 4°C, in the presence of protease inhibitors, and purification by isopycnic centrifugation. Two major PG, a proteochondroitin sulfate and a proteodermatan sulfate, were recovered. The chemical and physical properties of the proteochondroitin sulfate are described here.

**EXPERIMENTAL PROCEDURES**

**Materials**—Adult pig skin and bovine nasal septa were obtained frozen from Pel-Freeze Biologicals and kept at -15°C. Guanidine HCl (grade 1) and 6-aminoheptanoic acid were purchased from Sigma. Cesium chloride, technical grade, was obtained from Kawecki Berylco Ind., Revere, MA. Benamidine-HCl was a product of Matheson, Coleman and Bell. All other chemicals were of reagent quality. Standard preparations of glycosaminoglycans were gifts from Dr. Martin Mathews, University of Chicago, and a sample of hyaluronic acid was provided by Dr. D. A. Swann, Harvard Medical School. Sepharose 2B and 6B were from Pharmacia. Papain (twice crystallized) was from Worthington, and Alcian blue was from Eastman. Streptomyces hyaluronidase, chondroitinase AC and ABC, and chondroitin 4-sulfatase and 6-sulfatase were obtained from Miles Laboratories.

**Analytical Procedures**—Hexuronic acids were measured by a borate-containing carbazole method (10). except that all reagents were mixed before heating, and heating was for 15 min in a 100°C block. Carbazole/orcinol ratios were determined according to the method of Hoffman et al. (11). DNA was measured by absorbance at 290 nm and by the diphenylamine reaction (12). The amino acid composition of samples was determined on a Technicon TSM amino acid analyzer after hydrolysis in 6 N HCl at 110°C for 22 h in sealed, evacuated tubes. Hexosamines were measured on the longer column of the analyzer as described by Kieras (13), after hydrolysis in 4 N HCl at 100°C for 8 h. Protein was measured either by the Lowry method

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The abbreviation used is: PG, proteoglycan(s).
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Hydroxyproline was determined as described by Woesener (15).

Extraction of Proteoglycans—Pig skin was freed of fat and hair, cut into small pieces, frozen in liquid N₂, and pulverized in a Wiley Mill with a 20-mesh screen, cooled with liquid N₂. The powder was immediately lyophilized and extracted three times for 3 h each at 4°C with petroleum ether (16), freed of organic solvent, and stored at -15°C.

An initial extraction was made with 0.1 M NaCl in 0.05 M sodium acetate, pH 5.8 (15 ml/g of powder), stirred for 3 h at 4°C. This solvent and all further solvents, as far as associative and dissociative density gradient centrifugation were used. Proteolytic inhibitors: 0.1 M 6-aminohexanoic acid, 0.01 M ethylenediaminetetraacetic acid, and 0.005 M benzamide hydrochloride (17). The suspension was centrifuged at 23,500 × g for 30 min at 4°C, and the residue was further extracted, for exploratory purposes, with various concentrations of salts (30 ml/g of powder) at 4°C for 48 h with gentle rocking. The pH varied from 4 to 5.6 depending upon the salt present. Centrifugation at 25,900 × g for 45 min at 4°C gave a clear solution that was dialyzed against water and analyzed for uronic acid.

The procedure chosen (summarized in Fig. 1) was extraction with 0.1 M NaCl as above, then extraction of PG with 4 M guanidine HCl, 0.05 M sodium acetate, pH 5.8, for 48 h at 4°C (30 ml/g of powder), centrifugation, re-extraction with the same volume of solvent for 3 h at 4°C and centrifugation. The solutions were combined, and PG was precipitated from the guanidine solution by addition of 2.3 volumes of ethanol and KSCN as described by Toole and Lowther (8) for urea precipitations. The washed precipitate was suspended in water (6 to 7 ml/g skin powder) and dialyzed at 4°C against 0.05 M sodium acetate, pH 5.8, with proteolytic inhibitors. Addition of guanidine HCl, to a concentration of 4 M, then completed the dissolution of the precipitate.

As a control preparation, a pig skin sample that had been observed to contain little or no proteochondroitin sulfate (50 g) was mixed with 2 g of bovine nasal septum and pulverized and extracted exactly as described. This sample was carried through all subsequent steps for the isolation of proteochondroitin sulfate.

Association Density Gradient—PG solution was dialyzed against 9 volumes of buffer with inhibitors to reduce the guanidine concentration to associative conditions (18). Cesium chloride was added to a density of 1.05 g/ml (some cloudiness usually appeared). After centrifugation at 84,100 × g, at 16°C for 64 h in a Beckman-Spinco model L1-65B rotor, a thin gelatinous layer was lifted from the surface, and the gel was divided into six nearly equal frustules by aspiration from the top. These were labeled A6 to A1 from top to bottom (19). The fractions were thoroughly dialyzed against 0.05 M sodium acetate, pH 5.8, analyzed for uronic acid, hexosamine, and protein, and digested with papain for electrophoresis of glycosaminoglycans.

Dissociative Density Gradient—The A1 fractions from a density gradient were combined and diluted with approximately 0.5 volumes of water, and guanidine HCl was added to 4 M and CsCl to a density of 1.5 g/ml (20). The final volume was one-fourth to one-third of the original volume in the associative gradient. Centrifugation was repeated as before, and gradients were divided into six fractions (A1-D6 to A1-D1) and analyzed as above.

Column Chromatography—Analytical columns (0.9 × 136 cm) of Sepharose 2B or Sepharose 6B were equilibrated and eluted with 0.5 M sodium acetate, pH 7.0, or 4 M guanidine HCl, 0.05 M Tris-HCl, pH 7.0. Flow rates were 6 ml/h, and fractions of 1 ml were collected. V₀ was determined with highly polymerized calf thymus DNA or tritium-labeled λ phage DNA, and Vₐ with glucuronolactone. Sepharose 2B was calibrated with freshly prepared bovine nasal PG monomers and subtracted (10) and used with standard glycosaminoglycans. Samples contained 0.5 to 1 mg of uronic acid in 1 ml. Kᵥ values were determined by the equation of Laurent and Killander (21).

Characterization of Glycosaminoglycans—Samples of PG in 0.5 M sodium acetate, 0.1 M potassium phosphate, 0.01 M sodium EDTA, and 0.01 M cysteine, pH 6.5, were added to papain suspension that had been activated for 60 min at 37°C in 2 volumes of the same solution (papain/PG 1:125). After 18 h at 37°C, the digests were dialyzed against 50 to 100 volumes of 0.2 M sodium chloride and then against large volumes of distilled water with stirring. If necessary, samples were clarified by centrifugation for cellulose-acetate electrophoresis or chromatography on Sepharose 6B.

Cellulose-acetate electrophoresis of 0.5-μl samples of glycosaminoglycan (1 to 2 mg/ml) was carried out on the PhoroSlide medium (Millipore Biomedica, Acton, MA) in the cadmium acetate system of Shively and Conrad (22) at 1 mA per inch of width for 2 h. Stips were stained in Alcian blue according to the method of Newton et al. (23), but without ethanol. If concentration was necessary, glycosaminoglycans were precipitated with 3 volumes of 0.5 M sodium acetate in ethanol, kept at 4°C overnight, pelleted, washed with ethanol, dried under vacuum, and dissolved in water.

Enzymatic and Chemical Treatment of Proteoglycan—The proteochondroitin sulfate (A1-D1) was treated with Streptomyces hyaluronidase (24), which attacks only hyaluronic acid. PG (24 μg of uronic acid) was treated with 20 turbidity reducing units of enzyme in 250 μl of 0.1 M sodium acetate, pH 5.0, at 60°C for 6 h, and for a further 10 h with another 20 turbidity reducing units of enzyme in 100 μl. The digest was exhaustively dialyzed against the same buffer, and HCl was added for hydrolysis and hexosamine determination. For comparison, 24 μg of hyaluronic acid was similarly digested. All digested samples were related to controls in which enzyme was omitted.

For the determination of susceptibility to chondroitinases (25), digestions were done in 0.1 M sodium acetate, 0.1 M Tris-HCl, pH 7.3 (26). Samples of PG containing 50 μg of uronic acid in 200 μl were dialyzed against the buffer and digested with 0.1 unit of chondroitinase AC or ABC at 37°C for 1.5 h. An additional 0.1 unit of enzyme and a drop of toluene were added, and digestion was continued for 20 h. Digests were exhaustively dialyzed against 0.1 M sodium acetate, and HCl was added for hydrolysis and hexosamine determination for comparison against undigested controls.

The ratio of chondroitin 4-sulfate to 6-sulfate in the proteochondroitin sulfate was determined by Method II of Saito et al. (27). Since there was no evidence of dermatan sulfate in this PG, chondroitinase ABC was not used. Digestion mixtures contained chondroitinase AC and 6-sulfatase, with or without 4-sulfatase. A sample of the final disaccharide product, AD₃₄₅₆, was used as a standard in the Morgan-Elson colorimetric measurement.

Nitrous Acid Treatment—The low pH nitrous acid procedure of Shively and Conrod (28) was used to test for heparan sulfate or heparin structures in the PG. At room temperature 400 μl of HNO₃ was added to a solution of AI-D1 (45 μg of uronic acid) in 100 μl of water and to a reference solution of 250 μg of heparin. After 15 min, samples were dialyzed exhaustively against 0.01 M KCl, and HCl was added for hydrolysis and determination of hexosamines. Treated
solutions were compared to controls from which \( \text{HNO}_3 \) was omitted. 

**β Elimination and Reduction of PG—Proteochondroitin sulfate** (Fraction A1-D1) used for β elimination experiments and for amino acid analyses contained 2.43 mmol/ml of hexosamine (93% GluN, 7% GlcN) and 2.55 mmol/ml of uronic acid. For all experiments including controls, PG equivalent to 300 µg of uronic acid was used. Samples were treated with 0.5 M NaOH at 33°C for 24 h and also with alkaline borohydride (0.1 M NaOH, 0.6 M NaBH₄, 45°C, 24 h) and PdCl₂ by the procedure of Downs et al. (29), and in some instances the PdCl₂ was omitted. They were then hydrolyzed for amino acid analysis together with untreated controls. No corrections were made for destruction during hydrolysis.

**Binding of Proteoglycan Fractions to Hyaluronic Acid—** Binding studies were carried out essentially by the procedure of Hardingham et al. (30, 31). Samples of proteoglycan (A1 or A1-D1) containing 0.8 to 1.2 mg of uronic acid in 0.8 to 1.5 ml of associative buffer (0.5 M sodium acetate, pH 7.0) were mixed with solutions of hyaluronic acid (molecular weight in excess of 0.5 x 10⁸) in ratios of 25:1 to 400:1 (PG/hyaluronic acid). Mixtures were kept at 4°C for 60 min and then chromatographed on Sepharose 2B in the same buffer. Experiments were also carried out at 22°C, but no difference was found. As a control, PG monomer (A1-D1) prepared from bovine nasal septum in the presence of skin was combined with hyaluronic acid in the ratio 125:1 (31), and chromatographed on Sepharose 2B.

**RESULTS**

**Extraction of Proteoglycans—** Fig. 2 shows the proportion of total tissue hexuronic acid recovered from skin powder by various salt solutions. An initial extraction with 0.1 M NaCl removed 15 to 20% of the uronic acid, mostly as hyaluronic acid. Only a small amount of galactosamine was removed, the ratio of glucosamine to galactosamine being 11:1. The residue was extracted at 4°C with various solvents, each of which had a different optimal concentration. Guanidine HCl (4 M) was the most efficient solvent, since it removed about 55% of the remaining uronic acid. Urea at 6 M was almost as effective. Inorganic salts extracted only 30 to 40% of total uronic acid at their optimum concentration of about 1 M. Lanthanum chloride was less efficient at higher concentration, a kind of effect seen previously with calcium extraction of cartilage (18).

There were also differences in the ratio of galactosamine to glucosamine in the extracts. With guanidine HCl, urea, and most of the salts, the ratios were 1.3 to 1.5, whereas with LiCl the ratio was 0.27. A 48-h extraction period was optimal in each case, longer extractions solubilizing more collagen with little increase in PG. With guanidine HCl, extraction was independent of pH between 5 and 8. The observations, as a whole, are similar to those of Sajdera and Hascall (18) on extraction of cartilage, except for the somewhat lower extractability of skin PG.

In the procedure finally used (Fig. 1), a preliminary extraction with 0.1 M NaCl removed some of the easily extractable glucosamine and some noncollagenous protein (Fraction a, Table I). Cellulose acetate electrophoresis (Fig. 3) showed that this glucosamine was mainly in hyaluronic acid, but not all of the hyaluronic acid is easily extracted, as will be seen below. Subsequent extraction with 4 M guanidine HCl removed 71% of the total glucosamine, with substantial amounts of glucosamine and collagen (Fraction b, Table I). We used this fraction as representative of the galactosamin-containing PG (those containing chondroitin sulfate and der- matan sulfate). Some samples of skin gave PG extracts that contained no detectable chondroitin sulfate (Sample b, Fig. 3), but other portions of skin contained both dermatan and chondroitin sulfates, and these were used for the preparation of proteochondroitin sulfate described here. The presence of hyaluronic acid and heparan sulfate in the extract may account largely for the glucosamine content.

The residue after extraction was characterized following proteolytic digestion. It contained much of the total glucosamine (Fraction c, Table I), mostly as hyaluronic acid and heparan sulfate (Fig. 3). Hyaluronic acid resistant to extraction has been reported also in bovine aorta (7), and this may

![Table 1](http://www.jbc.org/)

| Fraction | Uronic acid (mg/g dry powder) | Glucosamine (mg/g dry powder) | Galactosamine (mg/g dry powder) | Total protein (mg/g dry powder) | Collagen (mg/g dry powder) |
|----------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|---------------------------|
| a. 0.1 M NaCl                  | 0.398                         | 0.035                         | 14.3                          | 0.12                         |
| b. 4 M GuHCl                   | 0.877                         | 0.543                         | 24.7                          | 26.9                         |
| c. Residue*                    | 0.012                         | 0.111                         |                               |                             |
| Sum                               | 2.10                         | 1.172                         | 6.9                           |                             |
| Whole powder*                   | 2.35                         | 1.69                          | 0.76                          |                             |
| d. 4 M GuHCl                   | 0.961                         | 0.758                         | 47.3                          | 33.0                         |
| e. Ethanol-KSCN                  | 0.724                         | 0.027                         | 0.46                          | 7.4                          |

*Sum of amino acid analysis.

b. Calculated from hydroxyproline analysis (×7.5).

c. GuHCl, guanidine HCl.

d. Dialyzed papain digest.

![Fig. 2](http://www.jbc.org/)

**Fig. 2.** Extraction of proteoglycans from pig skin-defatted powder with guanidine HCl, urea, and inorganic salt solutions of various concentrations. The uronic acid solubilized, including the amount extracted initially with 0.1 M NaCl (---), is given as per cent of total tissue hexuronic acid. All solutions contained protease inhibitors. Guanidine HCl (○) and urea (■) were in 0.05 M sodium acetate, pH 5.8. Solutions of LiCl (▲), CaCl₂ (△), MgCl₂ (☆) and LaCl₃ (□) were not buffered (pH between 4 and 5.5).

![Fig. 3](http://www.jbc.org/)

**Fig. 3.** Composite diagram of cellulose-acetate electrophoretic patterns of papain digests of (a) 0.1 M NaCl extract, (b) 4 M guanidine HCl extract, (c) residue. A6 through A1 are associative gradient fractions, and A1-D1 is the densest fraction of the dissociative gradient of A1. The standard mixture contained 0.5 µg of each component; HA, hyaluronic acid; HS, heparan sulfate; DS, dermatan sulfate; and CS, chondroitin 4- or 6-sulfate.
indicate a mechanical trapping or a chemical interaction with collagen or some other matrix component. The galactosamine in the residue (15% of total) was not studied further.

**Purification of Proteoglycan—**Attempts to fractionate the 4 M guanidine HCl extract directly by associative density gradient centrifugation resulted in poor resolution and irreproducible distributions of components. Much PG was bound or entrained in the collagenous layer at the surface. The PG, therefore, was freed of most of the extractable collagen by precipitation with ethanolic KSCN (8). The effect of this step is shown in Table I. An extract, Sample e, similar to Extract b, but somewhat richer in PG and protein, was treated with ethanolic KSCN as described under “Experimental Procedures.” In the precipitate, Sample e, 59% of the galactosamine was recovered, and there was a substantial reduction of protein and especially of collagen. The yield of PG was rather variable in the step, but the procedure was included because it greatly improved the following density gradient fractionation.

PG prepared as above was centrifuged in a CsCl density gradient, first under associative conditions so that aggregate forms of PG, if present, might be preserved and detected by gel chromatography. Fig. 4A shows the distribution of several components in the resulting six fractions. These values, with those in Table II, indicated that 30% of the uronic acid was recovered in Fraction A1 together with more than half of the galactosamine. Only chondroitin sulfate could be seen on cellulose acetate electrophoresis of a papain digest of A1 (Fig. 3). Very little of the total protein was present here, as would be expected if this fraction contained a proteochondroitin sulfate of high polysaccharide content and consequently of high buoyant density.

Dermatan sulfate was found in all fractions of the gradient (Fig. 3) except the densest, although mainly in the upper three. This suggests a protein-rich structure consistent with the 60% protein previously reported for proteodermatan sulfate (9). Heparan sulfate appeared in Fractions A2, A3, and A4, and might represent a separate PG as observed by others in aorta (6) and in lung (32). Hyaluronic acid was spread among all fractions except A1. Although these rather diffuse distributions might suggest various degrees of interaction among some of the PG and hyaluronic acid under associative conditions, no specific binding of proteochondroitin sulfate to hyaluronic acid could be demonstrated, as shown below. Measurements of DNA by absorbance at 260 nm, by the diphenylamine reaction (12), and by the carbazole method (10), indicated that enough DNA was present in Fractions A3 and A4 (Table II) to account quantitatively for the apparent high ratio of uronic acid to hexosamine.

Fraction A1 was further purified in a dissociative density gradient (Fig. 4B and Table II). About 82% of the uronic acid of Fraction A1 was recovered in the bottom two fractions, but only the densest fraction (A1-D1) was analyzed in detail. The ratio of glucosamine to galactosamine was lower than that of A1, possibly because of the removal of traces of hyaluronic acid or glycoprotein. The nature of the remaining glucosamine is considered later. A small amount of protein, present in A1, appeared in the upper fractions of the dissociative gradient (Fig. 4B), where there was very little uronic acid. From the amino acid analysis of the PG (A1-D1) and on the assumption that it contained 30% uronic acid, a protein content of 4 to 5% was calculated for proteochondroitin sulfate.

On Sepharose 2B (Fig. 5A), the PG moved with a $K_v$ of 0.53, and PG of bovine nasal septum, with a $M_r$ of $2.5 \times 10^6$ (33), had a $K_v$ of 0.18. On the assumption of similar hydrodynamic properties of the two PG, the skin product would have an $M_r$ of approximately $10^6$. Evidence for its PG structure is found in a comparison of its gel chromatographic behavior before and after papain digestion. Undigested A1-D1 was totally excluded from a column of Sepharose 6B, but after digestion the resulting chondroitin sulfate chains could be compared with a series of standard glycosaminoglycans (Fig. 6). The logarithms of the $M_r$ of the standards were plotted against their elution volumes, and the $M_r$ of the skin chondroitin sulfate was graphically found to be 20,000, the same as that of cartilage chondroitin sulfate (34). In addition, derman sulfate chains resulting from papain digestion of Fraction A6 were found to have an $M_r$ of 26,000 (Fig. 6).

**Aggregation of Proteoglycan—**In PG extracted from hyaline cartilage (20), Fraction A1 contains largely aggregates, and Fraction A1-D1 consists of monomers from which hyaluronic acid and link proteins have been dissociated and removed. In the control experiment PG of bovine nasal septum was isolated in the presence of skin in an associative density gradient and was chromatographed on Sepharose 2B (Fig. 5,
TABLE II

Analyses of fractions of associative and dissociative gradients

| Sample | Uronic acid % distribution in gradient | Glucosamine ratio in fraction | Galactosamine | GlcN/ GalN | Uronic acid/hexosamine | Density g/ml |
|--------|----------------------------------------|-----------------------------|---------------|---------|-----------------------|-------------|
| Associative gradient fraction | | | | | | |
| A6 | 17.6 | 47.6 | 16.6 | 1.80 | 0.78 | 1.56 |
| A5 | 12.2 | 16.4 | 8.5 | 1.21 | 1.33 | 1.60 |
| A4 | 16.4 | 10.7 | 5.5 | 1.21 | 2.74 | 1.64 |
| A3 | 13.5 | 8.5 | 5.9 | 0.90 | 2.46 | 1.68 |
| A2 | 9.7 | 6.6 | 9.7 | 0.30 | 1.44 | 1.73 |
| A1 | 30.6 | 10.1 | 53.8 | 0.12 | 1.04 | 1.77 |
| Dissociative gradient fraction | | | | | | |
| A1-D6 | 2.2 | 20.0 | 3.3 | 1.53 | 0.31 | 1.43 |
| A1-D5 | 2.1 | 7.4 | 2.2 | 0.84 | 0.61 | 1.47 |
| A1-D4 | 3.6 | 6.0 | 3.5 | 0.43 | 0.83 | 1.52 |
| A1-D3 | 5.6 | 5.4 | 5.6 | 0.24 | 0.93 | 1.57 |
| A1-D2 | 11.2 | 11.7 | 10.6 | 0.28 | 0.76 | 1.62 |
| A1-D1 | 71.7 | 10.0 | 70.3 | 0.09 | 1.09 | 1.66 |

% recovery from A1 above

| Sample | Uronic acid | Glucosamine | Galactosamine | GlcN/ GaIN | Uronic acid/hexosamine | Density g/ml |
|--------|-------------|-------------|---------------|---------|-----------------------|-------------|
| A1-D1  | 2.2         | 20.0        | 3.3           | 1.53    | 0.31                  | 1.43        |
| A1-D5  | 2.1         | 7.4         | 2.2           | 0.84    | 0.61                  | 1.47        |
| A1-D4  | 3.6         | 6.0         | 3.5           | 0.43    | 0.83                  | 1.52        |
| A1-D3  | 5.6         | 5.4         | 5.6           | 0.24    | 0.93                  | 1.57        |
| A1-D2  | 11.2        | 11.7        | 10.6          | 0.28    | 0.76                  | 1.62        |
| A1-D1  | 71.7        | 10.0        | 70.3          | 0.09    | 1.09                  | 1.66        |

Fig. 5. Sepharose 2B chromatography of proteochondroitin sulfate from skin and from bovine nasal septum (BNS) prepared in the presence of skin. Aggregate Fractions A1 (- - -) and monomer fraction A1-D1 (- - -) were eluted with (A) 0.5 M sodium acetate, pH 7.0, and (B) 4 M guanidine HCl, 0.05 M sodium acetate, pH 7.0. Bovine nasal septum monomer, when combined with hyaluronic acid, was indistinguishable from bovine nasal septum aggregate A1.

bovine nasal septum A1). Most of the PG appeared in the excluded volume as expected of an aggregated preparation (30, 31), indicating that the isolation procedure had neither removed bound hyaluronic acid nor damaged the binding region of the PG. A monomer, prepared from this A1 in a dissociative gradient, was retarded with a Kav of 0.18 (Fig. 5, bovine nasal septum A1-D1). Addition of hyaluronic acid to this material largely restored the aggregated form and moved the elution position back to the excluded volume.

In contrast, in the skin proteochondroitin sulfate, the molecular sizes of A1 and A1-D1 were essentially the same (Fig. 5), whether chromatographed under associative or dissociative conditions, and there was no evidence of aggregate. The addition of various proportions of hyaluronic acid to A1 or A1-D1 from skin had no effect on the position of their peaks. In this regard the skin proteochondroitin sulfate differs from that of cartilage.

Chemical and Enzymatic Analyses—The amino acid composition of proteochondroitin sulfate (A1-D1) is given in Table III together with analyses of other PG for comparison. There is a general resemblance to the composition of PG from bovine nasal cartilage, with the major difference that no sulfur-containing amino acids were detected in the skin product. Proteodermatan sulfate of skin, however, as analyzed by Öhrink (9), has a substantially different distribution of amino acids.

Additional evidence for the presence of chondroitin sulfate as the glycosaminoglycan in the A1-D1 preparation was pro-
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provided by the carbazole/orcinol ratio (11). The ratio was 1.20 for A1-D1, 1.39 for a chondroitin 4-sulfate standard, and 0.31 for a dermatan sulfate standard. This value, the essentially complete digestibility of the galactosamine-containing component by chondroitinase AC (Table IV), and the electrophoretic pattern (Fig. 3), constitute good evidence for the identity of the chondroitin sulfate, although the total absence of iduronic acid cannot be claimed.

In the proteochondroitin sulfate, about 8% of the hexosamine is glucosamine (Table III). If the PG contains only conventional glycosaminoglycans, this could indicate the presence of hyaluronic acid, heparan sulfate, keratan sulfate, or heparin, either as a contaminant or as part of the PG structure. Attempts were made to define the nature of the glucosamine-containing component by specific enzymatic and chemical treatments. Digestion with hyaluronidase caused little change in the hexosamine content, whereas under the same conditions a hyaluronic acid control was almost entirely destroyed (Table IV). Digestion with chondroitinase AC or ABC had no effect on the glucosamine, although galactosamine, as a part of chondroitin sulfate, was lost as expected. Compounds that contain N-sulfate groups, such as heparan sulfate and heparin, would be split by nitrous acid (28), and some material of this nature may be present since some of the glucosamine was lost under conditions that caused complete destruction of a heparin control (Table IV). At present no clear decision can be made about the nature of the glucosamine, but heparan sulfate and keratan sulfate are possible sources, the former because it is seen in the tissue (Fig. 3), and the latter because it would be resistant to the treatments listed in Table IV.

Linkage of Polysaccharide to Protein—The β elimination reaction provides evidence for glycosidic linkage of carbohydrates to proteins (35). A preliminary treatment of Fraction A1-D1 with 0.5 M NaOH at 23°C for 24 h caused destruction of 53% of the threonine and 66% of the serine. The loss of serine is consistent with the presence of a xylosyl-serine linkage of chondroitin sulfate to protein as described in cartilage PG by Lindahl and Rodén (36). The loss of threonine was unexpected because attachment of chondroitin sulfate chains to protein has generally been thought to be exclusively through serine (37). The elimination was therefore repeated with the inclusion of borohydride and PdCl₂ as described by Downs et al. (29), who reported that 2-amino-2-butenic acid residues produced from serine and threonine were quantitatively reduced to alanine and α-amino-2-butyric acid. On treatment of Fraction A1-D1, 55% of the threonine and 76% of the serine residues were destroyed (Table V). The reduction of unsaturated amino acid residues was, however, not quantitative, since only 72% of the threonine destroyed was converted to α-amino-2-butyric acid, and 53% of the serine destroyed was converted to alanine. If the PdCl₂ catalyst was omitted (results not shown), the destruction of threonine and serine remained the same, conversion of serine to alanine was the same, but conversion of threonine to α-amino-2-butyric acid was only 53%.

Analysis of Chondroitin Sulfates—The use of specific enzymes (27) allowed the quantitation of chondroitin 4- and 6-sulfates in the PG. The proportion in Fraction A1-D1 was 84.7% 4-sulfate and 15.3% 6-sulfate; values for Fraction A1 were similar (89.5% 4-sulfate). Nonsulfated portions of the polysaccharide would be included in the 6-sulfate estimation.

**DISCUSSION**

The extractability of PG from pig skin was measured with various salt solutions and dissociative solvents, of which 4 M guanidine HCl, the most effective, removed 71% of the galactosamine of the whole tissue (Table I). Two distinct types of PG were obtained by sedimentation in density gradients, one of low buoyant density and high protein content (proteo-dermatan sulfate) and one with a density greater than 1.7 g/ml (proteochondroitin sulfate). The yield of the latter (Fraction A1-D1) was 27% of the galactosamine of the dry skin powder, or about 0.75 mg of PG/g.

The proteochondroitin sulfate contained 4 to 5% of protein, and after papain digestion only chondroitin sulfate could be seen on cellulosic-acetate-acrylase A analyses (Fig. 3). This glycosaminoglycan had an M₆ of 20,000 and a content of 4-sulfate of 84%, like that of bovine nasal cartilage (38). About 8% of the galactosamine was glucosamine, not derived from hyaluronic acid, some of which might have come from traces of heparan sulfate since this compound was apparent in adjacent gradient fractions (Fig. 3) and since part of the glucosamine was lost on treatment with nitrous acid (Table IV). Some might also have arisen from keratan sulfate, as it does in cartilage PG (35), or from oligosaccharides resembling keratan sulfate (17). In cartilage, keratan sulfate is linked to protein partly through threonine residues, and this could explain the loss of threonine as well as serine on β elimination of skin proteochondroitin sulfate (Table V). Other types of glucosamine-containing oligosaccharides, like those found in corneal PG (39), might also be present.

Skin proteochondroitin sulfate does not bind to hyaluronic acid, as shown by the absence of aggregate in Fraction A1 from the associative gradient (Fig. 5) and by the lack of interaction of Fraction A1 or A1-D1 with added hyaluronic acid. It is unlikely that this is the result of damage to a binding region, since cartilage PG prepared by the same route in the presence of skin shows normal aggregation. The skin product, although smaller, resembles cartilage PG in having a low protein content and in its general amino acid composition (Table III). The absence of detectable cysteine, however, is a striking difference and may be related to the lack of binding

**Table IV**

| Treatment       | Proteoglycan (A1-D1) | Controls |
|-----------------|---------------------|----------|
|                 | GalN | GlcN | GalN | GlcN |
| Hyaluronidase   | 97   | 115  | 8    |      |
| Chondroitinase AC | 3    | 105  | 15   | 18   |
| Chondroitinase ABC | 7    | 100  | 12   | 15   |
| Nitrous acid    | 101  | 57   | 1    |      |

**Table V**

**β Elimination of proteoglycan**

Analysis of certain amino acids in Fraction A1-D1 before and after treatment with 0.1 M NaOH, 0.6 M NaBH₄, and PdCl₂ at 45°C for 24 h (98).

| Component            | Before | After | Destruction | Reduced product µmole/g |
|----------------------|-------|-------|-------------|------------------------|
| Threonine            | 28.7  | 12.9  | 15.8 (55%)  | 11.3                   |
| α-Aminobutyric acid  | 0     | 11.3  |             |                        |
| Serine               | 73.5  | 17.5  | 61.8 (78%)  | 32.8                   |
| Alanine              | 31.5  | 64.3  |             |                        |

**Table VI**

| Component            | Before | After | Destruction | Reduced product µmole/g |
|----------------------|-------|-------|-------------|------------------------|
| Threonine            | 28.7  | 12.9  | 15.8 (55%)  | 11.3                   |
| α-Aminobutyric acid  | 0     | 11.3  |             |                        |
| Serine               | 73.5  | 17.5  | 61.8 (78%)  | 32.8                   |
| Alanine              | 31.5  | 64.3  |             |                        |
to hyaluronic acid, since cystine disulfide linkages are essential to the functional integrity of the binding region of cartilage PG (4).

A speculative model of the proteochondroitin sulfate can be offered. A molecule of 10^6 daltons would have about 50 chains of chondroitin sulfate attached to serine residues. More than enough serine was lost on β elimination to account for these (62 mol/mol, Table V). The loss of threonine (16 mol/mol) might have resulted from the presence of keratan sulfate-like oligosaccharides as in PG of chondrosarcoma (17).

At present there is no evidence for binding of PG to hyaluronic acid, since cystine disulfide linkages are essential to the functional integrity of the binding region of cartilage (4). To bind to hyaluronic acid, since cystine disulfide linkages are essential to the functional integrity of the binding region of cartilage (4). Attempts have been made to extract proteochondroitin sulfate (Al, Table II) than proteodermatan sulfate extracted under much more drastic conditions. The large size of his product may have resulted from irreversible changes during extraction. Toole and Lowther (7) and Öbrink (8) also noted some chondroitin sulfate in dense gradient fractions, but did not characterize it as PG. The present observation in skin of two PG with very different composition and sizes raises questions of their roles in the molecular organization of the tissue.

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