Isolation and Characterization of Proteoglycans from Bovine Aorta*

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Proteoglycans from bovine aorta were extracted by 0.15 M NaCl and by digestion with collagenase and with elastase. The nonspecific protease activity of elastase was inhibited by soybean trypsin inhibitor. Glycosaminoglycans (GAG) of the proteoglycans were isolated, characterized, and quantitated. About 50% of the tissue chondroitin 4- and 6-sulfates, 75% of hyaluronic acid, and almost all chondroitin were extracted from the tissue by 0.15 M NaCl. Collagenase solubilized most of the chondroitin sulfates and part of the dermatan sulfate of the aorta. Elastase solubilized most of the heparan sulfate of the tissue and a large amount of dermatan sulfate. Sequential extraction of the tissue by NaCl, collagenase, and elastase yielded proteoglycans with varied GAG and protein compositions. Proteoglycans extracted by 0.15 M NaCl contained 13.2% protein and 18.5% hexuronic acid with 85% chondroitin 6-sulfate and 15% chondroitin 4-sulfate of the total GAG. Collagenase-solubilized proteoglycans from NaCl-extracted residue had 10.3% protein and 20.6% hexuronic acid with 77% chondroitin 6-sulfate, 14% chondroitin 4-sulfate, and 9% dermatan sulfate of the total GAG. The major proteoglycan obtained from collagenase-extracted residue by hydrolysis with elastase contained exclusively heparan sulfate with a protein content of 15.2%. Although the precise association of these proteoglycans with other constituents of the connective tissue of the arterial wall is not clearly established, it is suggested that chondroitin sulfate-dermatan sulfate proteoglycan is bound to collagen, and heparan sulfate proteoglycan to elastin.

The connective tissue of arterial wall is composed of a family of complex carbohydrates which are important in maintaining the integrity of the tissue. Although there is much information on glycosaminoglycans from arteries (1), little is known of proteoglycans, the native state in which GAG occur in the tissue. Fransson and Havsmark (2) isolated a dermatan sulfate proteoglycan from horse aorta and studied its structure. Their studies suggested that GAG of this proteoglycan contained chondroitin sulfates as well as dermatan sulfate in a polymeric structure. Kresse et al. (3) isolated a chondroitin sulfate-dermatan sulfate proteoglycan from bovine aorta maintained in culture and studied its physicochemical properties. Recently, Eisenstein et al. (4) isolated a proteoglycan from steer aorta capable of producing antibodies when injected into rabbits.

We previously (5) extracted proteoglycans from pooled bovine aorta by various disassociative solvents by the procedure of Sajdera and Hascall (6). These studies indicated that only about 50% of the total hexurionate contained in the tissue was isolated by this procedure. The hexuronate extracted by disassociative methods is part of a proteoglycan containing chondroitin sulfates as well as dermatan sulfate. No heparan sulfate was obtained although aorta contains a considerable amount of this GAG. This paper describes GAG and their association with other components, particularly collagen and elastin of the tissue. Since it is difficult to precisely quantitate individual proteoglycans obtained by different extraction procedures because of their polydispersity, we approached the problem by first quantitating individual GAG of each extract and then isolating individual proteoglycans in a highly purified form to study their composition. Bovine aorta was digested with elastase and collagenase, and GAG were isolated from the digests, characterized, and quantitated. Intact proteoglycans solubilized by collagenase and chloatoac were also isolated and their composition studied.

EXPERIMENTAL PROCEDURES

Materials

Fresh bovine aortas were obtained from a local abattoir and packed in ice for transport to the laboratory. The aortas were dissected free of extraneous tissue, minced into small pieces, and maintained at -20°. The tissue was dry-defatted by cold acetone (4°) over several changes. It was then pulverized in a micromill at -20°, and the pulverized tissue was stored in a desiccator at 4° until used for analysis.

Chromatographically purified collagenase (425 units/mg, Clostridium histolyticum), porcine pancreatic elastase (110 units/mg), orcinol-dyed elastin, and soybean trypsin inhibitor (twice crystallized) were purchased from Worthington Biochemical Corp. (Freehold, N. J.). Hyaluronidase (bovine testicular, 11,000 units/mg) was obtained from Nutritional Biochemicals Corp. (Cleveland, O.). Samples of GAG standards were generous gifts from Dr. J. A. Cifonelli (University of Chicago).

Analytical Methods

Total uronic acid in GAG was determined by the Dische method (7) or Bitter and Muir method (8), and hexosamine by the Boas method (9), omitting the use of resin after the sample had been...
hydrolyzed with 4 N HCl for 16 h. Total sulfate was determined by the
Dodson and Spencer method (10), as modified by Muir (11). After
the sample had been hydrolyzed by 12 N formic acid for 24 h, N-
Acetylhexosamine was determined according to the method of Reis-
sig et al. (12). Determination of N-acetyl groups (13) and differential
determinations of glucuronic acid and iduronic acid (14) and glucose-
amine and galactosamine (15) were performed by gas-liquid chroma-
tography as described previously. We determined N-sulfate groups
in heparan sulfate proteoglycan by the procedure of Lagunoff and
Warren (16), and measured protein content in proteoglycans accord-
ing to the method of Lowry et al. (17). Amino acid analyses were
performed on a Technicon amino acid analyzer after hydrolysis of the
sample with 6 N HCl for 24 h. Hydroxyproline was determined by the
Woessner method (18) after the sample had been hydrolyzed with 6 N
HCl for 3 h at 130°.

Hydrolysis of GAG by Hyaluronidase

The percent of GAG which could be hydrolyzed by hyaluronidase
was determined by digesting 1.0-mg samples with 100 units of hyalu-
ronidase in 23 ml of 0.1 M sodium acetate buffer, pH 5.3, containing
0.15 M NaCl at 37° for 24 h. At the end of 24 h, 50 units more of
hyaluronidase were added and the hydrolysis continued for another
24 h. The enzymatic activity was then stopped by heating the digest
in a boiling water bath for 5 min. The reaction mixture was cooled
in room temperature (24°) and exhaustively dialyzed against distilled
water over several changes. Uronic acid content in the nondialyzie-
able material was determined. GAG solutions without enzyme (blanks)
were treated similarly. From the difference in the uronic acid con-
tent between the experimental and blank, we calculated the per cent of
GAG hydrolyzed in the sample.

The proportion of chondroitin 4-sulfate to chondroitin 6-sulfate in
mixtures was estimated after the samples had been hydrolyzed by hyaluronidase by the procedure of Mathews and Inouye (19).

Digestion of Aorta by Elastase

Pulverized, dry-defatted aorta (1.0 g) was digested with 320 units
of elastase at 37° for 48 h in 25 ml of 0.2 M Tris/HC1 buffer, pH 8.8.
Thymol (15 mg) or penicillin (50,000 units)/streptomycin (50,000 µg)
was used to inhibit bacterial activity. Since elastase is known to
possess some proteolytic activity, it was inhibited by soybean trypsin
inhibitor as described by Walford and Kickhofen (20). In some exper-
iments elastolytic activity of the elastase preparation was inhibited by
increasing the NaCl concentration in the incubation mixture (20).
In control experiments aorta was extracted with the buffer, omitting
the enzyme. Aliquots of the digestion mixture were withdrawn at
different time intervals and liberation of amino nitrogen was deter-
mined by ninhydrin reaction (21).

Digestion of Aorta with Collagenase

Digestion of 1.0 g of dry-defatted aorta was achieved with 100 units
of collagenase in 25 ml of 0.05 M Tris/HC1 buffer, pH 7.5, containing
0.06 M CaCl2. The conditions of hydrolysis were similar to those
described above for digestion with elastase.

Hydrolysis of Serum Albumin and Elastin

Bovine serum albumin (25 mg) and orcine-dyed elastin (25 mg)
were hydrolyzed by 80 µg of elastase in the presence of 940 µg of
soybean trypsin inhibitor or 1.15 M NaCl, in the buffer system
previously described, at 37°. Controls were run without inhibitors.
At the end of 8 h the enzyme activity were determined either by
ninhydrin reaction in the case of albumin or by reading absorption at
590 nm in the case of orcine-dyed elastin. From the difference be-
 tween control and experimental values, per cent inhibition was

calculated in each case.

To determine whether protease activity was present in collagen-
ase preparations, we incubated bovine serum albumin (25 mg) with
2.50 µg of collagenase in 25 ml of 0.05 M Tris/HC1 buffer, pH 7.5,
containing 0.06 M CaCl2 at 37° for 8 h. The reaction was stopped by
the addition of 0.5 ml of 50% trichloroacetic acid. After centrifuga-
tion, aliquots of the supernatant were determined for the amino
nitrogen. No detectable amount of amino nitrogen was found in the
supernatant.

Isolation and Characterization of Glycosaminoglycans

After hydrolysis with the enzymes, the digestion mixture was
centrifuged and GAG were isolated from the supernatant by the
usual procedure (22, 23). Briefly, this consisted of adding NaOH to
the supernatant to a concentration of 0.1 N and allowing it to stand at
5° for 18 h; this was followed by precipitation of peptide material
with trichloroacetic acid, filtration through Celite, dialysis, and concen-
tration to a small volume. Uronic acid was then determined as a
measure of isolated GAG. The GAG mixture was then fractionated
on Dowex 1-C18 (200-400 mesh) by eluting with a stepwise increasing
concentration of NaCl 0.5 to 4.0 M (24). The Dowex 1-C18 column fractionation separates GAG into groups of:
hyaluronic acid is eluted in 0.5 M NaCl, chondroitin in 0.5 to 0.75
M NaCl, heparan sulfate in 1.0 to 1.5 M NaCl, chondroitin 6-
and 4-sulfates and dermatan sulfate in 1.5 to 2.0 M NaCl, and
heparin in 2.0 to 4.0 M NaCl. Variations occur depending upon
the source of GAG, and analytical methods are necessary to quanti-
tate the individual GAG. The fractions were exhaustively dialyzed,
concentrated to a small volume, and analyzed for uronic acid and
hexosamine contents, and individual GAG were quantitated. The
GAG fractions were further identified by hydrolysis with bovine
testicular hyaluronidase and electrophoresis on cellulose acetate
strips in pyridine/formic acid buffer pH 3.5 (25).

0.15 M NaCl and 3.0 M MgCl2 Extraction of Aorta

Aorta was separately extracted by 0.15 M NaCl and 3.0 M MgCl2
repeatedly (three times) until the extracts were negative to carba-
zole reaction (7) for hexuronic acid. The residual tissues were ex-
haustively washed with distilled water until free from Cl-. The
washed residues were digested by elastase and collagenase as previ-
ously described and GAG in the digests were studied.

Total GAG from Aorta

Total GAG of the tissue was determined by procedures previously
described (22, 23).

Isolation of Proteoglycans by Sequential Extractions of Tissue

In order to study the nature of proteoglycans released by the
effect of the enzyme, the tissue was sequentially extracted by 0.15 M
NaCl, collagenase, and elastase (Fig. 1).

0.15 M NaCl-soluble Proteoglycans—Dry-defatted bovine aorta (50
g) was repeatedly (three times) extracted with 0.15 M NaCl at 37° in
the presence of 0.6% penicillin/streptomycin. The combined extrac-
tives were exhaustively dialyzed against 0.15 M NaCl at 4°. To the di-
alyzed extract we added 2.5 volumes of ethanol and 1% (w/v) potas-
sium acetate and allowed the mixture to stand overnight (16 h) at 4°.
The flocculent precipitate produced was collected by centrifugation
at 6,000 × g for 1 h at 5°. It was washed and dehydrated with ethanol
and acetone and dried over vacuum.

Collagenase-solubilized Proteoglycans—The residue remaining
after extraction with 0.15 M NaCl was digested with 5,000 units of
collagenase at 37° for 24 h (penicillin-streptomycin was added to the
incubation media to inhibit bacterial growth). The digestion was
stopped at 6,000 × g and the residue was rehydrolyzed by
3,000 units of collagenase. The supernatants were pooled and after
exhaustive dialysis against 0.15 M NaCl at 4°, the proteoglycans
were precipitated from the supernatant as described earlier.

Elastase-solubilized Proteoglycans—The residue not digestible by
collagenase was hydrolyzed by 16,000 units of elastase for 24 h at 37°
in the presence of 0.8 g of soybean trypsin inhibitor. The digest was
centrifuged and the residue was rehydrolyzed by another addition
of 16,000 units of elastase at 37° in the presence of 0.8 g of soybean trypsin inhibitor. The digest was
centrifuged and the residue was rehydrolyzed by another addition
of 16,000 units of elastase. The supernatants from the two digestes
were combined, dialyzed against 0.15 M NaCl, and proteoglycans were
precipitated by adding ethanol as previously described.

Chromatography on Bio-Gel P-60—The crude proteoglycan
samples were dissolved in 0.1 M KC1 and centrifuged at 25,000 × g for
30 min at 4°. Small, insoluble residues which sedimented were dis-
carded and 3-ml aliquots of clear supernatants were chromatogram-
phgraphed on a Bio-Gel P-60 column (25 × 40 cm), eluting with 0.1 M
KC1. The eluates were collected in 3-ml fractions and assayed for
trichloroacetic acid. Those containing uronic acid, all in the void
volume, were pooled and concentrated by a collodion membrane filter
(75 µm) at 4° to a final volume of approximately 10 ml.

CM-cellulose Column Chromatography—The concentrated solu-
tions of proteoglycans obtained after Bio-Gel P-60 column were ex-
haustively dialyzed against 0.005 M sodium acetate buffer, pH 4.8 at
4°, and centrifuged to remove small amounts of insoluble material.
The clear supernatants were then chromatographed on a CM-cellu-
lose column (25 × 25 cm) by successive elution with 150 ml each of
sodium acetate buffer, pH 4.8, ionic strength 0.5, and the same buffer

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Bovine Aorta Proteoglycans

Aorta (dry-defatted)

Extract: 0.15 M NaCl, 37°, 24 h (3 times)

Centrifuge 6000 × g

Supernatant

NaCl-soluble proteoglycans

and proteins

Hydration: Collagenase, 37°, 94 h (2 times)

Centrifuge 6000 × g

Supernatant

Collagenase-solubilized proteoglycans;
collagen peptides

Hydration: Elastase, 37°, 24 h (2 times)

Centrifuge 6000 × g

Supernatant

Elastase-solubilized proteoglycans; elastin peptides

Sediment

Hydration: Elastase, 37°, 24 h (2 times)

Centrifuge 6000 × g

Supernatant

Elastase discards

Sediment

(>1% hexuronate; discard)

Fig. 1. Procedure of isolation of proteoglycans from bovine aorta.

The time course hydrolysis of aorta by elastase with and without protease inhibitor is shown in Fig. 2. Three different concentrations of soybean trypsin inhibitor were used, resulting in a gradual decrease in the hydrolysis of aorta with increasing concentration of the inhibitor. No further inhibition was observed above 16 mg concentration of the inhibitor in the system, i.e. enzyme/inhibitor ratio of 1:4. At this concentration of the inhibitor, 95% of protease activity of the elastase preparation was inhibited when bovine serum albumin was used as a substrate, but complete dissolution of orcinol dyed elastin occurred in 8 h. Since elastolytic activity of the enzyme can be inhibited by increasing the concentration of NaCl in the incubation media, we used two concentrations of NaCl. 0.65 and 1.15 M (20) (Fig. 2). Greater inhibition was observed at 1.15 M NaCl concentration than at 0.65 M. At 1.15 M NaCl concentration, about 85% of elastolytic activity of the enzyme was inhibited when orcinol dyed elastin was used as a substrate. At concentrations ranging from 1.5 M NaCl to 2.0 M NaCl, we observed no further inhibition of hydrolysis of either aorta or orcinol dyed elastin.

Alkaline Treatment of Proteoglycans—The proteoglycan samples (10 mg) were dissolved in 0.5 ml of 0.5 M NaOH and incubated at 5° for 24 h. The solution was then neutralized with acetic acid and dialyzed exhaustively against distilled water. Aliquots were hydrolyzed by 6 M HCl and analyzed for amino acids.

RESULTS

Digestion of Aorta by Elastase

The time course hydrolysis of aorta by elastase with and without protease inhibitor is shown in Fig. 2. Three different concentrations of soybean trypsin inhibitor were used, resulting in a gradual decrease in the hydrolysis of aorta with increasing concentration of the inhibitor. No further inhibition was observed above 16 mg concentration of the inhibitor in the system, i.e. enzyme/inhibitor ratio of 1:4. At this concentration of the inhibitor, 95% of protease activity of the elastase preparation was inhibited when bovine serum albumin was used as a substrate, but complete dissolution of orcinol dyed elastin occurred in 8 h. Since elastolytic activity of the enzyme can be inhibited by increasing the concentration of NaCl in the incubation media, we used two concentrations of NaCl. 0.65 and 1.15 M (20) (Fig. 2). Greater inhibition was observed at 1.15 M NaCl concentration than at 0.65 M. At 1.15 M NaCl concentration, about 85% of elastolytic activity of the enzyme was inhibited when orcinol dyed elastin was used as a substrate. At concentrations ranging from 1.5 M NaCl to 2.0 M NaCl, we observed no further inhibition of hydrolysis of either aorta or orcinol dyed elastin.

Analysis of GAG obtained after alkaline hydrolysis of the digests showed that hydrolysis of aorta by elastase without inhibitors solubilized about 22% of the total hexuronate material of the tissue, but in the presence of protease inhibitor at the concentration of maximum inhibition, only about 70% of total hexuronate was solubilized. When the elastolytic activity of the enzyme was inhibited by 1.15 M NaCl, only 55% of the
tic activity was inhibited most of the heparan sulfate and acid in the fractions, along with hydrolysis by hyaluronidase, mine and galactosamine, and glucuronic acid and iduronic. It is interesting that 40% of total GAG were extracted by Tris/HCl buffer, whereas only 75% of the total chondroitin was obtained in the extraction media. The GAG fractions were characterized and quantified by extensive analysis on Dowex 1-Cl- columns and analyses of the fractions are shown in Table I. The GAG fractions were characterized and quantified as shown in Table II, based on the contents of glucosamine and galactosamine, and glucuronic acid and iduronic acid in the fractions, along with hydrolysis by hyaluronidase and electrophoretic identification. Elastase in the presence of protease inhibitor released most of the GAG from the tissue except 50% of the total chondroitin sulfates, but when elastolytic activity was inhibited most of the heparan sulfate and dermatan sulfate were not obtained in the extraction media. Hyaluronic acid and chondroitin were obtained from all of the digests. Tris/HCl buffer extracted about 75% of the total hyaluronic acid, 40% of the chondroitin sulfate, and most of the chondroitin.

**Digestion of Aorta by Collagenase**

In previous studies (5), a further hydrolysis by collagenase after 3.0 M MgCl2 extraction and then digestion by elastase solubilized little or no GAG. In the present studies, aorta without prior treatment was digested with collagenase and GAG were studied in the digest in order to find the nature of GAG released by this hydrolysis. Time course of hydrolysis is shown in Fig. 2. Hydrolysis proceeded rapidly during the first 24 h and afterwards we observed no appreciable increase in ninhydrin values. GAG isolated from the digest is reported in Tables I and II. GAG were quantified by extensive analysis of the fractions obtained after fractionation of crude GAG on the Dowex 1-Cl- columns. Hydrolysis of aorta by collagenase solubilized all GAG except heparan sulfate and dermatan sulfate.

**Digestion of NaCl- and MgCl2-extracted Aorta by Enzymes**

In a follow-up experiment, the dry-defatted aorta was repeatedly (three times) extracted separately with 0.15 M NaCl and 3.0 M MgCl2 until no more hexuronic were obtained in the extraction media, then digested by the enzymes to study what activity of the enzyme. For comparison, hydrolysis of the aorta by elastase in the presence of 16.0 mg of soybean trypsin inhibitor and by collagenase were performed simultaneously (right). Tissues hydrolyzed by elastase without inhibitors and with inhibitors (16.0 mg of soybean trypsin inhibitor and 1.15 M NaCl) and by collagenase were used to study GAG released by the enzyme.

GAG could be released from the residual tissues by these enzymes. Analysis of pooled NaCl and MgCl2 extracts of the tissue showed that 0.70 and 1.1 mg of hexuronate/g of tissue were extracted by 0.15 M NaCl and 3.0 M MgCl2, respectively.

Time course of the hydrolysis of the residual tissue by collagenase and elastase is shown in Fig. 3. There was no difference in the time course of hydrolysis by elastase between NaCl- and MgCl2-extracted residues, but a great difference was observed in the time course hydrolysis by collagenase. The rate of hydrolysis of MgCl2-extracted residue was considerably lower than that of NaCl-extracted residue, and comparatively less total hexuronic was obtained from these residual tissues than from tissue without prior treatment. Hexuronic acid was not detected in some of the eluates of the Dowex 1-Cl- columns used for the fractionation of GAG (Table III). The composition of GAG isolated from these digests is shown in Tables III and IV. Eighty to ninety-five per cent of total hexuronic acid was obtained in the time course hydrolysis by elastase. About 5 times more dermatan sulfate was obtained by elastase digestion than by collagenase. Collagenase, on the other hand, solubilized 7 to 8 times more chondroitin sulfates than elastase, while no heparan sulfate was identified in collagenase digests. The presence of hexuronic acid in all digests is interesting.

**Isolation and Purification of Proteoglycans**

Table V shows the yields and compositions of proteoglycans extracted by 0.15 M NaCl and solubilized by collagenase and by elastase. Since the materials contained large amounts of contaminating protein and peptides, hexuronic acid could not be determined directly. The hexuronic acid values for the total GAG isolated from the crude proteoglycans agreed with those previously described. After the digestion of the tissue with elastase a small amount of tissue remained, which was observed in our previous studies (5) to have only small amounts of contaminating protein and peptides, hexuronic acid could not be determined directly. The hexuronic acid values for the total GAG isolated from the crude proteoglycans agreed with those previously described. After the digestion of the tissue with elastase a small amount of tissue remained, which was observed in our previous studies (5) to have only small amounts of GAG (less than 1% of total hexuronic acid of the tissue). We did not attempt, therefore, to extract GAG from this residue. After purification by gel filtration on Bio-Gel P-60 to eliminate low molecular weight peptides, the proteoglycans con-
### Table I

**Analyses of Dowex 1-Cl\textsuperscript{-} column fractions of bovine aorta GAG obtained by digestion with elastase and collagenase**

The abbreviations used are: GAG, glycosaminoglycans; UA, hexuronic acid; GlcUA, glucuronic acid; IdUA, iduronic acid; GlcN, glucosamine; GalN, galactosamine; HA, hyaluronic acid; CS, chondroitin sulfates; HS, heparan sulfate; DS, dermatan sulfate.

<table>
<thead>
<tr>
<th>Elastase (no inhibitor)</th>
<th>1,750</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elastase + protease inhibitor</td>
<td>1,350</td>
</tr>
<tr>
<td>Collagenase</td>
<td>1,200</td>
</tr>
<tr>
<td>Blank (no enzyme)</td>
<td>790</td>
</tr>
</tbody>
</table>

#### Fractions eluted at m NaCl

<table>
<thead>
<tr>
<th></th>
<th>0.5 + 0.75</th>
<th>1.0 + 1.25</th>
<th>1.5</th>
<th>2.0 + 4.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total GAG*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Elastase (no inhibitor)</td>
<td>450</td>
<td>340</td>
<td>600</td>
<td>190</td>
</tr>
<tr>
<td>GlcUA:IdUA</td>
<td>100:00</td>
<td>100:00</td>
<td>93:7</td>
<td>64:36</td>
</tr>
<tr>
<td>GlcN:GalN</td>
<td>88.9:11.1</td>
<td>100:00</td>
<td>6:94</td>
<td>27:73</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>HA</td>
<td>HS</td>
<td>CS, DS</td>
<td>HS</td>
</tr>
<tr>
<td>Hydrolysis by hyaluronidase (%)</td>
<td>94</td>
<td>0</td>
<td>88</td>
<td>75</td>
</tr>
<tr>
<td>Elastase + protease inhibitor</td>
<td>440</td>
<td>290</td>
<td>440</td>
<td>70</td>
</tr>
<tr>
<td>GlcUA:IdUA</td>
<td>100:00</td>
<td>100:00</td>
<td>84:16</td>
<td>57:43</td>
</tr>
<tr>
<td>GlcN:GalN</td>
<td>80.6:11.4</td>
<td>100:00</td>
<td>11.4:88.6</td>
<td>0.100</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>HA</td>
<td>HS</td>
<td>CS, DS</td>
<td>HS</td>
</tr>
<tr>
<td>Hydrolysis by hyaluronidase (%)</td>
<td>91</td>
<td>0</td>
<td>70</td>
<td>94</td>
</tr>
<tr>
<td>Collagenase</td>
<td>460</td>
<td>30</td>
<td>560</td>
<td>110</td>
</tr>
<tr>
<td>GlcUA:IdUA</td>
<td>100:00</td>
<td>100:00</td>
<td>98.5</td>
<td>100:00</td>
</tr>
<tr>
<td>GlcN:GalN</td>
<td>88:11</td>
<td>100:00</td>
<td>0.100</td>
<td>58:41</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>HA</td>
<td>HS</td>
<td>CS</td>
<td>DS</td>
</tr>
<tr>
<td>Hydrolysis by hyaluronidase (%)</td>
<td>90</td>
<td>0</td>
<td>88</td>
<td>94</td>
</tr>
<tr>
<td>Blank (no enzyme)</td>
<td>340</td>
<td>0</td>
<td>340</td>
<td>50</td>
</tr>
</tbody>
</table>

* Total hexuronate of the tissue obtained by alkali extraction followed by papain digestion was 1.9 mg/g of tissue.
* Fractions were pooled for analyses since only small amounts were eluted at 0.75, 1.25, 4.0 m NaCl.
* Elastase and collagenase were purified by chromatography on CM-cellulose, since the proteoglycan preparations contained considerable amounts of hydroxyproline, and the collagen peptides are known to bind to this ion exchange cellulose under the conditions of the experiment (26, 29). Most of the hexuronate-containing material in all the proteoglycan preparations was obtained during elution with the first buffer, i.e. acetate at pH 4.8, 0.5 ionic strength (Fig. 4). Although all the hexuronate from 0.15 m NaCl-extracted and collagenase-solubilized proteoglycans was eluted from the column as single peaks, elastase-solubilized proteoglycan preparations resolved into three peaks. The major peak fraction representing about 75 to 80% of the total hexuronate content was used for further studies. There was about 50% decrease in hydroxyproline content in 0.15 m NaCl-extracted material.

### Table II

**Composition of bovine aorta GAG obtained by digestion with elastase and collagenase**

GAG estimated from the analyses of the fractions from Dowex 1-Cl\textsuperscript{-} column reported in Table I.

<table>
<thead>
<tr>
<th></th>
<th>HA\textsuperscript{a}</th>
<th>Chon</th>
<th>HS</th>
<th>CS (6 4 SO\textsubscript{4})</th>
<th>DS</th>
<th>Hep\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elastase (no inhibitor)</td>
<td>400</td>
<td>50</td>
<td>380</td>
<td>670</td>
<td>115</td>
<td>50</td>
</tr>
<tr>
<td>Elastase + protease inhibitor</td>
<td>390</td>
<td>50</td>
<td>340</td>
<td>360</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Collagenase</td>
<td>410</td>
<td>50</td>
<td>30</td>
<td>590</td>
<td>40</td>
<td>45</td>
</tr>
</tbody>
</table>

* *The abbreviations used are HA, hyaluronic acid; Chon, chondroitin; HS, heparan sulfate; CS, chondroitin sulfates; DS, dermatan sulfate; Hep, heparin.
* Total of CS 6-SO\textsubscript{4} and 4-SO\textsubscript{4} are reported.
* Chon was calculated from GalN in 0.5 m NaCl elutes of the Dowex 1-Cl\textsuperscript{-} column, but not characterized.
* Hep was calculated from GlcN in the 2.0 + 4.0 m NaCl eluates of the Dowex 1-Cl\textsuperscript{-} column, but not characterized.
FIG. 3. Time course hydrolysis of bovine aorta after 0.15 M NaCl and 3.0 M MgCl₂ extraction with elastase and collagenase. In the course of hydrolysis by elastase, there was no difference between NaCl- and MgCl₂-extracted tissue. Collagenase did not hydrolyze the MgCl₂-extracted tissue as much as the NaCl-extracted aorta.

Table III
Analyses of Dowex 1-Cl⁻ column fractions of bovine aorta GAG obtained by digestion with elastase and collagenase preceded by 0.15 M NaCl and 3.0 M MgCl₂ extraction

<table>
<thead>
<tr>
<th></th>
<th>Total GAG</th>
<th>Fractions eluted at m NaCl</th>
<th>Fractions eluted at m MgCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.0 + 0.75*</td>
<td>1.0 + 1.25*</td>
</tr>
<tr>
<td>Residue of 0.15 M NaCl extraction</td>
<td></td>
<td>620</td>
<td>110</td>
</tr>
<tr>
<td>Elastase + protease inhibitor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GlcUA:IdUA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GlcN:GalN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>HA</td>
<td>HS</td>
<td>CS, DS, HS</td>
</tr>
<tr>
<td>Hydrolysis by hyaluronidase (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagenase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total UA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GlcUA:IdUA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GlcN:GalN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>HA</td>
<td></td>
<td>CS, DS</td>
</tr>
<tr>
<td>Hydrolysis by hyaluronidase (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blank (no enzyme)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total UA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Residue of 3.0 M MgCl₂ extraction

<table>
<thead>
<tr>
<th></th>
<th>Total GAG</th>
<th>Fractions eluted at m NaCl</th>
<th>Fractions eluted at m MgCl₂</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.0 + 0.75*</td>
<td>1.0 + 1.25*</td>
</tr>
<tr>
<td>Elastase + protease inhibitor</td>
<td></td>
<td>720</td>
<td>180</td>
</tr>
<tr>
<td>GlcUA:IdUA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GlcN:GalN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>HA</td>
<td>HS</td>
<td>CS, DS, HS</td>
</tr>
<tr>
<td>Hydrolysis by hyaluronidase (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagenase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total UA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GlcUA:IdUA</td>
<td></td>
<td>100:0</td>
<td>99:7</td>
</tr>
<tr>
<td>GlcN:GalN</td>
<td></td>
<td>100:0</td>
<td>100:0</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td>HA</td>
<td></td>
<td>CS, DS</td>
</tr>
<tr>
<td>Hydrolysis by hyaluronidase (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blank (no enzyme)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Fractions were pooled for analysis since only small amounts were eluted at 0.75, 1.0, and 4.0 M NaCl from the column.

** Chondroitin sulfates, dermatan sulfate, and heparan sulfate (CS, DS, and HS) have the same mobility in this system; tentative identification is based on the electrophoretic mobility and the nature of hexuronic acid (UA) and hexosamine. HIA, hyaluronic acid.

Further characterization not performed due to limitation of the amount of the sample.

Hexuronic acid not detected.

NaCl-extracted and collagenase-solubilized proteoglycans, but in elastase-solubilized proteoglycan only a small amount (0.02%) of hydroxyproline was observed (Table V) after CM-cellulose chromatography. However, this technique could not be expected to remove all of the collagen peptides, since some peptides may be bound to the proteoglycans by stronger ionic interactions than would occur with the weaker acidic groups of this ion exchange cellulose.

Using proteoglycan extracts from cartilage, Sajdera and Hascall (6) and others (30-33), have shown that CsCl density gradient centrifugation in the presence of 4.0 M guanidinium chloride effectively prevents association of collagen and other
TABLE IV
Composition of bovine aorta GAG obtained by digestion with elastase and collagenase preceded by 0.15 M NaCl and 3.0 M MgCl₂ extraction
GAG estimated from the analyses of the Dowex 1-Cl⁻ column fractions reported in Table III.

<table>
<thead>
<tr>
<th>Extraction</th>
<th>HA</th>
<th>HS</th>
<th>CS-4SO₄</th>
<th>DS</th>
<th>Hep</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residue of 0.15 M NaCl extraction</td>
<td>110</td>
<td>320</td>
<td>40</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Elastase + protease inhibitor</td>
<td>-</td>
<td>-</td>
<td>310</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>Collagenase</td>
<td>130</td>
<td>-</td>
<td>180</td>
<td>360</td>
<td>20</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Extraction</th>
<th>HA</th>
<th>HS</th>
<th>CS-4SO₄</th>
<th>DS</th>
<th>Hep</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residue of 3.0 M MgCl₂ extraction</td>
<td>130</td>
<td>-</td>
<td>180</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>Elastase + protease inhibitor</td>
<td>-</td>
<td>-</td>
<td>180</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>Collagenase</td>
<td>130</td>
<td>-</td>
<td>180</td>
<td>20</td>
<td>-</td>
</tr>
</tbody>
</table>

a The abbreviations used are: HA, hyaluronic acid; HS, heparan sulfate; CS, chondroitin sulfate; DS, dermatan sulfate; Hep, heparin; UA, hexuronic acid.

b Enzyme/protease inhibition = 1:4.
c Not detected.
d Calculated from GlcN in the 2.0 + 4.0 NaCl eluate of Dowex 1-Cl⁻ column but not characterized further.

TABLE V
Yields and composition of crude proteoglycans from bovine aorta obtained by sequential extraction

<table>
<thead>
<tr>
<th>Extraction</th>
<th>Yield</th>
<th>UA</th>
<th>Protein</th>
<th>Hydroxyproline</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15 M NaCl-extracted</td>
<td>mg/g</td>
<td>% dry weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>18.2</td>
<td>3.8</td>
<td>80.4</td>
<td>0.76</td>
</tr>
<tr>
<td>After Bio-Gel P-60</td>
<td>14.6</td>
<td>4.6</td>
<td>78.7</td>
<td>0.63</td>
</tr>
<tr>
<td>After CM-cellulose</td>
<td>12.2</td>
<td>5.5</td>
<td>69.5</td>
<td>0.29</td>
</tr>
<tr>
<td>Collagenase-solubilized</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>16.0</td>
<td>4.8</td>
<td>76.7</td>
<td>1.46</td>
</tr>
<tr>
<td>After Bio-Gel P-60</td>
<td>14.1</td>
<td>5.9</td>
<td>73.6</td>
<td>0.42</td>
</tr>
<tr>
<td>After CM-cellulose</td>
<td>8.7</td>
<td>9.1</td>
<td>62.4</td>
<td>0.25</td>
</tr>
<tr>
<td>Elastase-solubilized</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>89.5</td>
<td>0.5</td>
<td>95.4</td>
<td>0.56</td>
</tr>
<tr>
<td>After Bio-Gel P-60</td>
<td>26.1</td>
<td>1.7</td>
<td>82.8</td>
<td>0.49</td>
</tr>
<tr>
<td>After CM-cellulose</td>
<td>10.2</td>
<td>4.3</td>
<td>79.3</td>
<td>0.02</td>
</tr>
</tbody>
</table>

a Based on lyophilized dry weight.
b Hexuronic acid (UA) was determined on isolated GAG.

proteins with proteoglycans. In earlier studies of chondroitin sulfate-dermatan sulfate proteoglycan (5) we too observed that collagen and other proteins floated in the low density region of the gradient, whereas proteoglycans sedimented to the high density region. The CsCl density gradient centrifugation in the presence of 4.0 M guanidinium chloride was therefore used to further purify the proteoglycans. The sedimentation profiles of the proteoglycans obtained after CM-cellulose chromatography are shown in Fig. 5. In the 0.15 M NaCl-extracted proteoglycan preparation, more hexuronic acid and protein were observed in the low density region (d < 1.8) than in the high density region (d > 1.8). Collagenase-solubilized proteoglycans showed slightly more hexuronic acid in the bottom one-third of the gradient than in the top one-third, and the middle third of the gradient had the least hexuronic acid. Elastase-solubilized proteoglycans, in contrast, had the highest hexuronic acid in the middle region of the gradient, although the highest protein content was observed in the top third.

Electrophoresis of Proteoglycans—Fig. 6 shows the cellulose acetate electrophoretic patterns of proteoglycan fractions from

FIG. 4. Chromatography of crude proteoglycans on a CM-cellulose column (3.5 × 25 cm) after sequential extraction. Most of the hexuronate was eluted from the column in all the three preparations by the acetate buffer pH 4.8, ionic strength 0.5. Fractions under the major hexuronic acid peaks were pooled for further studies.

FIG. 5. CsCl density gradient centrifugation profiles of proteoglycans. The centrifugations were performed in a dissociative density gradient whose loading density was 1.06 g/ml. Uronic acid and protein content (absorbance at 280 nm) of each fraction was determined. Most of the hexuronate was found in the following fractions: in 0.15 M NaCl extract, bottom Fractions 1 to 3 and top Fractions 10 to 12; in collagenase-solubilized proteoglycans, bottom Fractions 1 to 4; and in elastase-solubilized proteoglycans, Fractions 3 to 8.
CsCl density gradient centrifugation after dialysis. Since several of the middle fractions from 0.15 M NaCl-extracted and collagen-solubilized proteoglycan failed to show any stain with Alcian blue due to low concentrations, they were concentrated approximately 5-fold prior to electrophoresis. In all preparations, Fractions 10 to 12 showed one spot with electrophoretic mobility similar to hyaluronic acid. Fractions 1 to 6 of 0.15 M NaCl-extracted proteoglycan showed only one spot each, and these migrated between standard samples of chondroitin sulfate and hyaluronic acid. Fractions 7 to 9 showed two components. Fraction 1 from collagenase-solubilized proteoglycans showed two spots. The faster migrating spot had mobility similar to a standard sample of heparin, and the other slower than chondroitin sulfates but faster than hyaluronic acid. Fractions 2 to 8 had one component with electrophoretic migration similar to the slower moving component of Fraction 1. Fraction 9 was a mixture of two components. Fractions 1 to 7 of elastase-solubilized proteoglycans appeared homogeneous and had mobility similar to Fractions 2 to 8 of collagenase-solubilized preparation; Fractions 8 and 9 showed two spots. Based on these results and the density gradient profiles, fractions that were homogeneous were studied further after being pooled as follows: Fractions 10 to 12 from each preparation, referred to hereafter as Fraction I of the respective preparation; tubes 1 to 6 of 0.15 M NaCl-extracted proteoglycans, Fractions 2 to 8 of collagenase-solubilized proteoglycans, and Fractions 3 to 7 of elastase-solubilized proteoglycans, referred to hereafter as Fraction II of the respective preparation; and Fractions 1 and 2 of elastase-solubilized proteoglycans, referred to as Fraction III. Due to limitation of material, certain studies could not be performed on the elastase-solubilized Fraction III.

Chromatography on Sephadex G-200—Since these proteoglycan fractions contained considerable amounts of protein that might have been a contaminant, the proteoglycan preparations were further purified on a Sephadex G-200 column calibrated with GAG of known molecular weights. The chromatographic elution profiles of different proteoglycan fractions are shown in Fig. 7. Fraction I from the three preparations was resolved into two or more protein peaks, but all hexuronic acid was eluted from the column as a single peak with little absorption at 280 nm. From the elution volumes of the hexuronic acid peaks, molecular weights of approximately 95,000 to 100,000 were estimated (Fig. 8). The other proteoglycan fractions were further purified on a Sephadex G-200 column (0.9 x 100 cm). Samples were eluted with 0.025 M NaCl. Fractions were analyzed for uronic acid and protein (absorbance at 280 nm). Void volume (V0) of the column was determined using Escherichia coli. Although multiple peaks were observed for proteins in some proteoglycan preparations, only single peaks were noted for hexuronates. Fractions corresponding to hexuronates were pooled for further studies. Elastase-solubilized proteoglycan Fraction III was not studied due to limitation of material.

![Fig. 6. Electrophoretic patterns of proteoglycan fractions obtained after CsCl density gradient centrifugation (see Fig. 5). The fractions were dialyzed against distilled water and analyzed by electrophoresis without any further treatment. Electrophoretic mobilities were compared with standard GAG (hyaluronic acid, HA; chondroitin 6-sulfate, CS; heparin, HEP). Electrophoresis was performed on cellulose acetate strip in pyridine/formic acid buffer (pH 3.3) 100 V, 20 min, Aelcian blue stain. Based on this analysis and CsCl density gradient centrifugation profiles, fractions which were homogeneous were pooled for further studies. For details refer to the text.](http://www.jbc.org/)

![Fig. 7. Gel filtration of proteoglycan fractions on a Sephadex G-200 column (0.9 x 100 cm). Samples were eluted with 0.025 M NaCl. Fractions were analyzed for uronic acid and protein (absorbance at 280 nm). Void volume (V0) of the column was determined using Escherichia coli. Although multiple peaks were observed for proteins in some proteoglycan preparations, only single peaks were noted for hexuronates. Fractions corresponding to hexuronates were pooled for further studies. Elastase-solubilized proteoglycan Fraction III was not studied due to limitation of material.](http://www.jbc.org/)

![Fig. 8. Molecular weight determination of proteoglycans by gel filtration on a Sephadex G-200 column (see Fig. 7). The column was calibrated with standard chondroitin 4-sulfate (CS-A), dermatan sulfate (DS), chondroitin 6-sulfate (CS-C), and chondroitin sulfate-dermatan sulfate proteoglycan from bovine aorta (CS-DS PG). A standard sample of hyaluronic acid was eluted in the void volume and is therefore not shown in the figure. Log molecular weights (M) were plotted against elution volume. Sample numbers on the curve refer to fractions, as follows: 1, 0.15 M NaCl-extracted Fraction I; 2, 0.15 M NaCl-extracted Fraction II; 3, collagenase-solubilized Fraction I; 4, collagenase-solubilized Fraction II; 5, elastase-solubilized Fraction I; 6, elastase-solubilized Fraction II.](http://www.jbc.org/)
The properties of proteoglycans from bovine aorta obtained by sequential extraction are shown in Table VI. Fraction II extracted by 0.15 M NaCl contained about 13% protein and about 18% hexuronic acid. Hexosamine was approximately equal in proportion to hexuronic acid. The proteoglycan contained only glucuronic acid. The proportion of chondroitin 6-sulfate to 4-sulfate was 17:3. The collagenase-solubilized Fraction II had somewhat less protein (10%) than 0.15 M NaCl-extracted Fraction II, and had both glucuronic and iduronic acids, although the hexosamine was mostly galactosamine (97%). From the iduronic acid value, the content of dermatan sulfate was estimated as 9% of the total GAG. The proportion of chondroitin 6-sulfate to 4-sulfate to dermatan sulfate was estimated as 33:6:4, a composition similar to that of the 3.0 M MgCl₂-extractable proteoglycan that we previously studied (5). The elastase-solubilized Fraction II had a protein content of 15%; glucosamine was present as the only hexosamine and was partly N-sulfated; and all uronic acid was present as glucuronic acid. Further, the proteoglycan was not digested by hyaluronidase, and the GAG of the proteoglycan was characterized as heparan sulfate. The elastase-solubilized Fraction III had a protein content of 12%; both glucosamine and galactosamine were present in the preparation. About 33% of the total uronic acid was found as iduronic acid, and both glucosamine and galactosamine were contained in a proportion of 3:1. The GAG composition was found to be chondroitin 6-sulfate, dermatan sulfate, and heparan sulfate.

The amino acid composition of the proteoglycan is shown in Table VII. In all preparations, large amounts of glycine, alanine, glutamic acid, and aspartic acid were present. In 0.15 M NaCl-extracted and collagenase-solubilized Fractions II, even after extensive purification, small amounts of hydroxyproline were found. Although no hydroxyproline, desmosine, and isodesmosine were detected in the elastase-solubilized Fraction II, two small unidentified peaks between phenylalanine and lysine were observed in the chromatogram of the amino acid analysis. The observed increase in proline could be due to degradation of glutamic acid.

**DISCUSSION**

Proteoglycans in the aorta are highly heterogeneous. Some can be easily extracted by salt solutions while others require solubilization of fibrous components of the tissue for extraction. Only about 50% of total hexuronic acid from aorta is extractable by the dissociative methods (6), while the remaining insoluble material requires additional extraction techniques. Although it is possible to extract most of the proteoglycan material from several cartilagenous tissues by dissociative techniques (6), these methods fail to isolate all proteoglycans from aortic tissue.

The large amount of proteoglycans present in an insoluble form could represent: (a) the existence of cross-linked complexes between proteoglycans and fibrous proteins; (b) the...
formation of more stable cross-linked complexes between several molecules of proteoglycans and hyaluronic acid with or without involvement of link proteins, (c) the formation of insoluble complexes by interaction of proteoglycans with collagen molecules of proteoglycans and hyaluronic acid with or without involvement of link proteins; (c) the formation of proteoglycan-collagen and -elastin cross-linkings play an important role in making proteoglycan material insoluble. If the insolubility were unrelated to specific interactions and reflected an intrinsic property of these proteoglycans alone, hydrolysis with these enzymes would not have further re-explained. Observations in our laboratory tend to rule this out. The specificity of these enzymes may be questioned since they may hydrolyze other components of the tissues. However, even highly purified preparations of elastase have some nonspecific protease activity, but this extraneous activity can be inhibited by soybean trypsin inhibitor (20). The observation of 96% inhibition of hydrolysis of bovine serum albumin in the presence of soybean trypsin inhibitor suggests the presence of only minimal nonspecific protease activity under the conditions of the studies. It is unlikely that this small amount of nonspecific protease activity could account for the large increase observed in ninhydrin values of aortic tissue hydrolysis by elastase with trypsin inhibitor.

Further, elastolytic activity may be considered not to be specific to elastin since other proteins containing alanine-lysine peptides may be hydrolyzed. But in aorta, elastin and collagen constitute the major bulk of the proteins, the former constituting about 60 to 70% of the dry-defatted tissue. Another fibrous protein that is closely related to elastin, although present in small amounts, is microfibrilar protein, or "structural glycoproteins," which Ross and Bornstein (35) have shown to be not hydrolyzable by elastase. The observation that only a small amount of lysine is present in the purified heparan sulfate proteoglycan suggests that this proteoglycan may not contain large numbers of alanine-lysine peptide residues. However, the nonextractable residue may contain some unidentified proteins susceptible to elastase activity, which might be associated with heparan sulfate proteoglycan. This point, therefore, remains open to question. In contrast, the studies with collagenase, which is highly specific and unlike elastase, did not show any nonspecific protease activity.

If we assume, for the present discussion, that these enzymes specifically hydrolyzed collagen and elastin under the conditions of the experiment, the following may be concluded: about 50% of chondroitin sulfates of the tissue and part of dermatan sulfate are bound to collagen, and heparan sulfate is bound to elastin in bovine aorta.

Sajdak and Hascall (8), Rosenberg et al. (36), Harding and Muir (37), and Hascall and Heinegard (39) have extensively studied aggregation of proteoglycans from cartilage in the presence of hyaluronic acid and link proteins. If somewhat more stable cross-linked complexes between proteoglycans and hyaluronic acid occur in bovine aorta, the resistance of these proteoglycans to extraction by salt solution might be explained. Observations in our laboratory tend to rule this out, as we have not observed the presence of such large aggregates in bovine aorta (5). Furthermore, Heinegard et al. (40) recently observed that in cartilage the core protein region close to keratan sulfate, but not close to the chondroitin sulfate region, binds hyaluronic acid to form an aggregate. This supports our finding since the presence of keratan sulfate in aorta has not been clearly established.

Other studies (41) from our laboratory show that sulfated GAG are capable of binding with serum low density lipoproteins in the presence of Ca" and Mg2+ to form insoluble complexes. In view of recent observations by Urry and coworkers (42) that Ca" binds to neutral sites of elastin and collagen, it is possible that the proteoglycans form insoluble complexes with collagen and elastin in the presence of Ca" and Mg2+ and are resistant to extraction with these salt solutions. Such complexes should be dissociable by solutions at

---

**Table VII**

<table>
<thead>
<tr>
<th>Components</th>
<th>0.15 M NaCl-extracted Fraction II</th>
<th>Collagenase-solubilized Fraction II</th>
<th>Elastase-solubilized Fraction II</th>
<th>μMole/mg proteoglycan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>0.104</td>
<td>0.086</td>
<td>0.115</td>
<td>0.112</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.060</td>
<td>0.045</td>
<td>0.032</td>
<td>0.028</td>
</tr>
<tr>
<td>Serine</td>
<td>0.068</td>
<td>0.045</td>
<td>0.049</td>
<td>0.026</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.080</td>
<td>0.060</td>
<td>0.117</td>
<td>0.091</td>
</tr>
<tr>
<td>Proline</td>
<td>0.054</td>
<td>0.054</td>
<td>0.086</td>
<td>0.083</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.111</td>
<td>0.112</td>
<td>0.032</td>
<td>0.049</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.148</td>
<td>0.160</td>
<td>0.267</td>
<td>0.274</td>
</tr>
<tr>
<td>Valine</td>
<td>0.092</td>
<td>0.080</td>
<td>0.235</td>
<td>0.223</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.047</td>
<td>0.041</td>
<td>0.133</td>
<td>0.129</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.079</td>
<td>0.034</td>
<td>0.090</td>
<td>0.083</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.101</td>
<td>0.010</td>
<td>0.011</td>
<td>0.015</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.094</td>
<td>0.034</td>
<td>0.029</td>
<td>0.020</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.035</td>
<td>0.022</td>
<td>0.032</td>
<td>0.031</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.044</td>
<td>0.026</td>
<td>0.011</td>
<td>0.013</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.021</td>
<td>0.009</td>
<td>0.008</td>
<td>0.006</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.052</td>
<td>0.032</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hydroxyproline*</td>
<td>&gt;0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0</td>
</tr>
</tbody>
</table>

* Determined by the procedure of Woessner (18).

* Not determined.
high salt concentrations, unlike the proteoglycans from bovine aorta.

The distribution of chondroitin sulfate proteoglycans both in the 0.15 M NaCl extract and the collagenase-solubilized medium suggests the presence of at least two species of chondroitin sulfate proteoglycans in the aorta. In the collagenase-solubilized medium, the proteoglycans contain considerable amounts of dermatan sulfate in addition to chondroitin sulfates. The composition of the proteoglycans is similar to a chondroitin sulfate-dermatan sulfate hybrid proteoglycan previously isolated from bovine aorta by extraction with 3.0 M MgCl₂ (5). Hybrid GAG are present in several tissues (43, 44) and in the proteoglycan from bovine aorta maintained in culture (3).

It is interesting that even after extensive purification the proteoglycans obtained by 0.15 M NaCl extraction and by collagenase solubilization of the residual tissue contain trace amounts of hydroxyproline. Whether this amino acid is an integral part of the proteoglycan or a contaminant is not possible due to unavailability of the material.

If the molecular weights of the proteoglycans calculated by the gel filtration method represent true molecular weights of these substances, the number of serine residues lost by alkaline hydrolysis tentatively suggests the presence of the following: five chains of GAG in 0.15 M NaCl-extracted chondroitin sulfate proteoglycan, four chains of GAG in collagen-solubilized chondroitin sulfate-dermatan sulfate proteoglycan; and five chains of GAG in elastase-solubilized heparan sulfate proteoglycan. All linked to the protein through serine.

Although we have yet to understand the precise nature of association of proteoglycans with collagen and elastin in bovine aorta, these observations clearly reveal specific binding between collagen and chondroitin sulfate-dermatan sulfate proteoglycan, and between heparan sulfate proteoglycan and elastin.

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REFERENCES
