Properties of Cartilage Proteoglycans†

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ABSTRACT: Proteoglycans extracted by dissociative means from porcine costal cartilage were fractionated on DEAE- and QAE-Sephadex into glycoprotein, collagen-like protein, and a spectrum of proteoglycans. The amino acid and amino sugar compositions and partial structures for this family of proteoglycans are described. The fractionation of purified proteoglycans by preparative scale disc gel electrophoresis, ultrafiltration, and gel-exclusion chromatography is also described. The proteoglycans isolated are macromolecules with slight but different amino acid compositions, contain no cysteine or methionine and appear to have varying proportions of chondroitin 4-sulfate and presumed keratan sulfate chains. The molecular weight distribution of these molecules has significant heterogeneity as well as possible association. Somewhat quantized molecular weights arise from varying degrees of saccharide chain substitution on identical or similar peptide cores or from variations in peptide length. The stoichiometry of saccharide chain substitution employing the elimination–sulfite addition reaction is reported and average saccharide chain molecular weights (14,000) calculated by this method agree well with figures obtained from sedimentation equilibrium studies of purified chondroitin sulfate chains.

The present knowledge of the structure and physical character of connective tissue proteoglycans[1] is due in part to the development of extraction and fractionation techniques which avoid chemical or enzymatic degradation and minimize the tendency of the covalent units to aggregate.

Early studies (Jorpes, 1929) resulted in the isolation of fractions exhibiting considerable heterogeneity. It has been generally assumed that the vigorous extraction procedures employed led to variability over and above that due to the presence of naturally occurring incomplete biosynthetic fragments and products arising from catabolic breakdown in the tissue.

Since the establishment of the covalent linkage between polypeptide and chondroitin sulfate chains (Anderson *et al.*, 1965), isolation procedures have avoided proteolytic digestion or exposure to alkali in an effort to maintain the native proteoglycan structure.

The proteoglycans obtained under such conditions are generally easy to free of collagen (Tsiganos and Muir, 1969) as evidenced by the absence of hydroxyproline in final products. Several reports (Tsiganos and Muir, 1967; Hoffman and Mashburn, 1967) have suggested that chondroitin sulfate and keratan sulfate chains are attached to the same peptide core but it has not been established that this is obligatory (universal) and the peptides themselves have not been characterized.

Cartilage proteoglycan, extracted by neutral salt, was partitioned into two fractions utilizing an isolation scheme where the products, termed protein–polysaccharides, were resolved according to their sedimentation characteristics (PPL[2] (light) and PPH (heavy) (Gerber *et al.*, 1960). Several subfractions of PPL were obtained by differential sedimentation (Pal *et al.*, 1966), but the relationship between PPL and PPH was not clarified.

A recent study (Sajdera and Hascall, 1969) surveyed various extraction techniques and concluded that the most effective (dissociative extraction) involves stirring cartilage slices in guanidinium chloride or $CaCl_2$ of high ionic strength which is buffered near neutrality.

Fractions isolated by dissociative techniques were claimed to be near native in structure and to interact with a glycoprotein whose function was to promote aggregation (Hascall and Sajdera, 1969). Attempts to resolve such preparations by differential sedimentation or electrophoretic techniques (Mashburn and Hoffman, 1971) have been reported. Although proteoglycan could be prepared free of collagen, homogeneity was not established and detailed composition and molecular weight data were not reported on fractionated samples. A detailed molecular weight study (Woodward *et al.*, 1972) has indicated that cartilage proteoglycan preparations may not only be heterogeneous as regards molecular weight but may associate as well.

This paper describes the extraction of proteoglycans by dissociative means from porcine costal cartilage and the fractionation of these extracts into glycoprotein, collagen-like protein, and a spectrum of proteoglycans containing slightly different amino acid composition with varying proportions of chondroitin 4-sulfate and presumed keratan sulfate chains. Compositions and partial structures for the family of compounds isolated are described. The overall composition of material extracted by dissociative methods is nearly identical with that extracted by disruptive procedures which consist of high-speed homogenization of the tissue in salt solutions of low ionic strength.

A comparison of the hexuronate contents of successive $CaCl_2$ extractions of cartilage slices indicate that the amino

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[1] The terminology employed to describe covalent structures containing carbohydrate and protein is extremely diverse. The following have been employed, more or less interchangeably, to identify molecules of this type isolated from cartilaginous tissue: chondromucoprotein, protein–polysaccharide, proteoglycan. The term proteoglycan will be employed in this manuscript to refer to this class of molecules. Generic terms such as glycoprotein, although applicable, usually refer to molecules predominantly protein in nature.

[2] Abbreviations used are: CS, chondroitin sulfate; C-4-S, chondroitin 4-sulfate; POPOP, 1,4-bis[2-(5-phenyloxazole)]benzene; PPH, protein–polysaccharide heavy; PPL, protein–polysaccharide light; PPO, 2,5-diphenyloxazole; Temed, *N,N,N′,N′*-tetramethylethylenediamine.

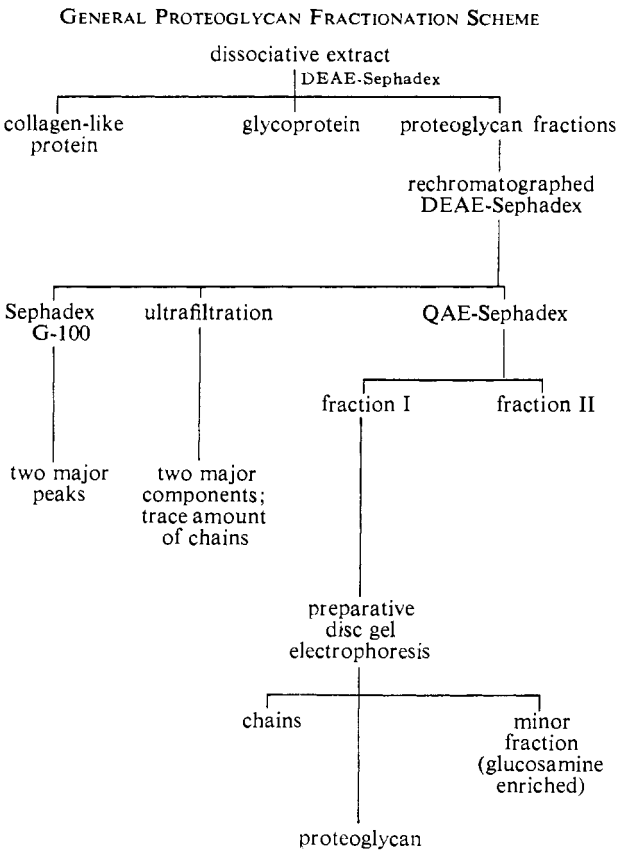

FIGURE 1: General outline for the fractionation of cartilage proteoglycans. Extraction was carried out with $CaCl_2$ or guanidinium chloride. Details are given in the text.

acid and amino sugar composition of sequential extracts are similar with an increase in the content of soluble collagen with further extraction.

Chromatography on DEAE-Sephadex A-50 was followed by rechromatography on the same medium. A glycoprotein fraction was isolated which contained cystine but in contrast, the major proteoglycan fraction and the peptide core derived from it by β elimination and sulfite addition (Simpson and Davidson, 1972), is free of sulfur-containing amino acids. The major proteoglycan fractions isolated showed negligible change in amino acid or amino sugar composition on refractionation. The major components were in turn chromatographed twice on QAE-Sephadex and, in general, two fractions were obtained. The first had a composition compatible with that of a C-4-S containing proteoglycan while the second appeared to have a significant increase in glucosamine and threonine content with a concomitant decrease in serine, indicative of keratan sulfate containing proteoglycans. The first peak represented about 70% of the material on a weight basis.

The major fractions were also examined by preparative discs gel electrophoresis, ultrafiltration, and gel-exclusion chromatography on Sephadex G-100. Molecular weight data are provided for several of the fractions obtained and the behavior of the purified fractions toward alkaline sulfite (Simpson *et al.*, 1972) is reported.

Materials and Methods

Chemicals used in this investigation were the best available commercially. Porcine costal cartilage was obtained as fresh as possible, carefully cleaned, and chopped into small slices which were extracted with a 15-fold amount (w/v) of 2.2 M $CaCl_2$. The starting pH was 6.4 and was maintained during extraction by periodic additions of 0.1 N NaOH. The extraction was carried out at room temperature for 48 hr, following which the suspension was filtered (or decanted) and the filtrate exhaustively dialyzed against water at 4°. The solution was filtered through washed Celite, and concentrated by ultrafiltration using an Amicon apparatus with a UM-10 membrane.[3] The retarded material was diluted with 0.4 M NaCl–4 M urea and reconcentrated three times in order to obtain a final solution approximately 0.4 M in NaCl and 4 M in urea.

Initial chromatography was performed on DEAE-Sephadex A-50 columns equilibrated in 0.4 M NaCl–4 M urea. An aliquot of the concentrated extract, generally containing about 1 g of proteoglycan, was placed on a 5 × 50 cm column and the column washed with 2 l. of starting salt solution. This eluate contained collagen-like protein with a characteristically high glycine and hydroxyproline content, and negligible amino sugar. Elution was continued with a series of 2-l. linear salt gradients ranging from 0.4 to 0.75 M NaCl, 0.75 to 1.0 M NaCl, and 1.0 to 1.5 M NaCl all in 4 M urea. Ten ml fractions were collected and proteoglycan elution was qualitatively monitored by spot testing every tenth fraction for Alcian Blue staining. Positive areas were analyzed for uronic acid content. When the gradient reached 1.5 M NaCl in concentration, the column was washed at room temperature with 1 l. of 1.5 M NaCl–8 M urea. The main peak and appropriate surrounding areas were combined, concentrated in the Amicon chamber, dialyzed against water, and lyophilized. Rechromatography on DEAE-Sephadex or QAE-Sephadex was carried out following the above scheme using columns of appropriate size based on the amount of proteoglycan to be fractionated. The overall fractionation scheme is summarized in Figure 1.

The analysis of samples for amino acids, and the amino sugars, glucosamine, and galactosamine, was performed on a Beckman Model 120C amino acid analyzer following hydrolysis *in vacuo* in 6 N HCl for 24 or 48 hr. Peak areas on chromatograms were determined using an on-line integrator and compositions were calculated by means of a computer program designed for that purpose and developed in this laboratory. Data from three standard runs was averaged to provide normalized peak areas. Fresh standards were run every week or whenever fresh ninhydrin was prepared. Appropriate corrections were made for the loss of serine and threonine during the hydrolytic procedure. Hydroxyproline analysis was also performed on the Beckman amino acid analyzer employing the appropriate standard.

Ultrafiltration experiments were performed using the Amicon Model 52 ultrafiltration chamber purchased from Scientific Systems Division, Amicon Corp., Lexington, Mass. Diaflo ultrafilters of the UM and XM series were employed in fractionation schemes. Specific details are provided in the appropriate result section.

Elimination–sulfite addition reactions were performed at pH 11.5 in the presence of freshly prepared 0.2 M sodium sulfite. The proteoglycan concentration was 1 mg/ml in both analytical and preparative-scale reactions. Optimal reaction conditions were established by examining the rate of cysteic acid formation as a function of pH and sulfite concentration.

[3] The general retentivity of Diaflo ultrafilters employed is: XM-100, greater than 100,000 molecular weight; XM-50, greater than 50,000 molecular weight; UM-10, greater than 10,000 molecular weight; UM-05, greater than 500 molecular weight.

Alkaline sulfite treated proteoglycan was resolved into peptide core and saccharide chains by chromatography on DEAE-Sephadex A-50. A column (2 × 45 cm) of DEAE-Sephadex A-50 was equilibrated with 0.4 M NaCl–4 M urea (pH 7.0). The elimination–sulfite addition reaction mixture was applied to the column in the same salt–urea solution. Peptide was washed from the column with a 600-ml volume of 0.4 M NaCl–4 M urea and routinely located by addition of ^{35}S-labeled marker peptide. The saccharide chains were eluted with a linear slat gradient (1200 ml) consisting of 0.4–1.5 M NaCl–4 M urea. Saccharide-containing fractions were located by spotting on paper and staining with Alcian Blue dye; positive fractions were then quantitated by the orcinol procedure, combined, deslated by dialysis against distilled water, and lyophilized.

Analytical acrylamide disc gel electrophoresis was carried out at a current of 2.5–3.0 mA/tube for 75 min in a Shandon electrophoretic cell SAE-2731. Gels consisted of 7.5% acrylamide and 3.6% bis (based on acrylamide content), and Temed; ammonium persulfate was employed to initiate polymerization. Gels were equilibrated in Tris–borate buffer (pH 9.5) and the dye marker was employed for staining proteins (Weber and Osborn, 1969).

Preparative-scale disc gel electrophoresis was performed in a Shandon preparative polyacrylamide electrophoresis apparatus SAE-2782 purchased from Shandon Scientific Co. Ltd., London. Gels were equilibrated in 0.2 M Tris–boric acid buffer (pH 8.5). Several agarose–acrylamide gel mixtures were employed ranging from 1 to 2.5% acrylamide. The fractionation results were independent of the composition of the gel. Samples were applied (50 mg of proteoglycan) dissolved in 5 ml of Tris–borate buffer to a small pocket (1 cm diameter) made by inserting a tube into the gelling liquid during polymerization. The upper outlet of this tube was stoppered with a cork which, after insertion, provided a liquid tight chamber in the middle of the future gel in which the sample was placed. Small fractions (1 ml) were collected due to the sharpness of the eluted peaks. The initial current was 30 mA which after 1 hr was increased to 80 mA for the duration of the run.

Uronic acid was determined by the orcinol method (Brown, 1946) or the carbazole method (Dische, 1947) and absorbance was recorded at 660 and 530 mμ, respectively.

Radioactivity measurements were made in a Packard Tri-Carb liquid scintillation counter employing an internal standard. Samples were spotted on paper disks and suspended in a solution of toluene containing PPO (4 g/l.) and POPOP (0.1 g/l.) as scintillators.

Molecular weights were determined by sedimentation equilibrium in a Beckman Model E analytical ultracentrifuge employing Rayleigh interference optics. Data were analyzed using a computer program generously donated by Dr. Dennis Roark of the department (Woodward *et al.*, 1972).

Results

The results of the DEAE-Sephadex chromatography for a typical extraction are summarized in Table I. The major fractions isolated represent better than 90% recovery of galactosamine and about 70% weight recovery of material placed on the column. The divisions between the fractions were arbitrary since the uronic acid containing material was eluted in a very broad peak comprising over 80 fractions. The amino acid composition of the fractions are very similar with slight differences in several of the amino acids. The absence of cystine and methionine was consistent. The overall composition suggests a lower proportion of peptide in the latter fraction; keratan sulfate is present in trace amounts, if at all. Fraction 2 was rechromatographed on DEAE-Sephadex and a single uronic acid containing peak was obtained which had essentially unchanged amino acid composition.

TABLE I: Composition of Major Fractions following DEAE-Sephadex Chromatography.[a]

Amino Acid[b]	Crude Extract	1	2	2 Rechromatographed
Lys	15.1	10.2	9.2	7.4
His	7.2	11.0	8.7	10.4
Arg	42.5	28.7	24.9	25.6
Asp	72.3	80.9	63.2	58.7
Thr	44.9	62.7	57.2	64.0
Ser	88.8	142.9	143.1	138.7
Glu	118.9	141.0	133.6	134.5
Pro	102.5	91.5	84.5	91.2
Gly	220.8	144.6	140.8	139.4
Ala	86.7	80.5	71.0	70.9
Cys				
Val	38.6	51.7	45.3	50.2
Met				
Ile	23.2	31.8	29.1	34.8
Leu	55.9	76.9	67.6	68.6
Tyr	12.0	17.8	12.6	15.5
Phe	23.6	28.1	21.9	23.3
Glucosamine[c]	22.7	32.5	35.7	49.9
Galactosamine	379.5	501.9	570.1	576.1
Quantity (mg)	1183 (750 mg used for column)	355	151	

[a] Amino acid analysis of a typical cartilage extract, the two fractions after chromatography on DEAE-Sephadex, and the composition after rechromatography of the second fraction on DEAE-Sephadex. [b] Residues per 1000 residues peptide. [c] Residues per 1000 total residues.

An aliquot of the first DEAE-Sephadex fraction was subjected to chromatography on QAE-Sephadex. A broad uronic acid containing peak was divided into two fractions which differed somewhat in amino acid composition, although both were similar to the starting material. Both threonine and glucosamine were enriched in the second fraction. Similar results were obtained following QAE-Sephadex chromatography of the second DEAE fraction. Results are summarized in Table II.

The major QAE-Sephadex fraction was subjected to preparative-scale disc gel electrophoresis. About one-third of the material was recovered as saccharide chains containing only a small percentage of amino acids. The contents of serine, lysine, glutamic acid, glycine, and tyrosine were relatively high in this fraction. Results are shown in Table III. The elution profile of the gel fractionation (Figure 2) shows a very sharp peak for the initial fraction with the remainder of the material tailing quite broadly. Analytical disc gel patterns revealed no

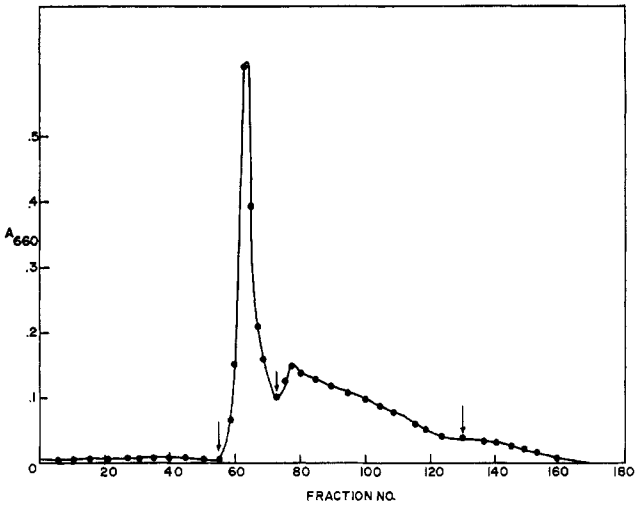

FIGURE 2: Elution pattern of material subjected to preparative disc gel electrophoresis. The sharp peak, combined as indicated by the first two arrows (fractions 56–72), represents C-4-S chains with a very small peptide residue attached. The second, broad, peak was combined as indicated (fractions 73–126) and represents a spectrum of proteoglycan molecules. A third fraction did not enter the gel. Fractions of 1 ml were collected and analyzed for uronic acid content by the orcinol reaction. For analytical details, see Table III.

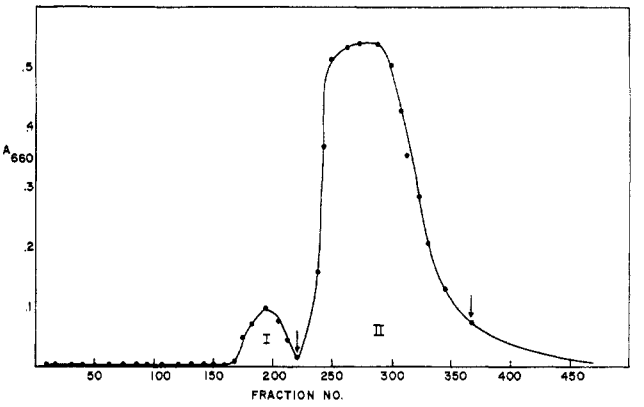

FIGURE 3: Elution profile of a typical DEAE-Sephadex chromatography of a $CaCl_2$ extract of cartilage. Peak I (fractions 163–219) has a composition similar to that of a glycoprotein but was not studied further. Peak II (fraction 221–360) is the main proteoglycan fraction. Fractions of 10 ml were collected and aliquots were analyzed for uronic acid by the orcinol reaction. For analytical details, see Table I.

significant mobility differences between the various fractions obtained.

In a second experiment, the material remaining insoluble after the 48-hr extraction period was reextracted twice for 24-hr periods with 2 l. of 2.5 M $CaCl_2$. The three extracts were analyzed separately and results are summarized in Table IV. The amino acid analyses clearly indicate that sequential extractions solubilize primarily collagen with about 80% of the galactosamine content appearing in the first extract. Analysis of remaining insoluble material is that expected for collagen. Following DEAE-Sephadex chromatography (Figure 3), the molecular weight distribution of the major fraction was determined by equilibrium ultracentrifugation. The results indicated significant size heterogeneity as well as possible association.

TABLE II: Amino Acid Composition of QAE-Sephadex Fractions.[a]

Amino Acid[b]	1	2
Lys	9.1	8.6
His	12.9	11.7
Arg	27.1	33.8
Asp	71.5	71.5
Thr	*52.4*	*74.7*
Ser	*156.9*	*133.9*
Glu	151.1	155.3
Pro	91.9	100.2
Gly	141.5	130.0
Ala	71.4	78.3
Cys		
Val	51.6	51.1
Met		
Ile	39.9	29.1
Leu	82.4	71.2
Tyr	18.4	20.9
Phe	23.0	31.4
Glucosamine[c]	*19.9*	*57.3*
Galactosamine	625.5	487.7
Quantity[d] (mg)	257	59

[a] Amino acid composition of QAE-Sephadex fractions. [b] Residues per 1000 residues peptide. [c] Residues per 1000 total residues. [d] Recovery was essentially quantitative.

TABLE III: Amino Acid Composition of Preparative Disc Gel Electrophoresis Fractions.[a]

Amino Acid[b]	Starting Material	1	2	3
Lys	9.1	53.0	29.7	4.7
His	12.9	20.0	11.6	8.4
Arg	27.1	6.4	30.5	31.4
Asp	71.5	66.1	74.4	95.5
Thr	52.4	34.0	53.0	55.9
Ser	156.9	213.2	174.1	151.8
Glu	151.1	155.7	143.1	150.1
Pro	91.9	53.0	80.9	69.2
Gly	141.5	186.1	149.2	141.0
Ala	71.4	75.2	90.1	80.0
Val	51.6	32.0	43.0	41.2
Ile	39.9	36.1	39.4	26.7
Leu	82.4	48.3	61.0	60.9
Tyr	18.4	16.5	13.0	18.2
Phe	23.0	6.0	22.1	27.0
Glucosamine[c]	19.9	4.8	18.9	26.3
Galactosamine	625.5	800.1	360.1	159.7
Quantity (mg)	75	23.6	30.4	11.7

[a] Amino acid analysis of fractions obtained after resolution by preparative disc gel electrophoresis of the major QAE-Sephadex fraction 1 (Table II). [b] Residues per 1000 residues peptide. [c] Residues per 1000 total residues.

TABLE IV: Amino Acid Analysis of Sequential Extracts of Cartilage.[a]

Amino Acid[b]	Extract 1	Extract 2	Extract 3
Lys	20.9	18.6	13.9
His	9.6	6.8	4.6
Arg	38.1	42.1	36.0
Asp	76.4	68.1	74.0
Thr	46.7	39.2	35.3
Ser	109.9	84.0	70.0
Glu	130.8	120.4	116.5
Pro	104.7	100.9	129.2
Gly	194.7	240.7	296.2
Ala	85.7	87.9	106.7
Val	44.6	31.9	29.2
Met	16.1		
Ile	29.2	63.8	17.9
Leu	61.9	66.7	44.4
Tyr	9.5	9.9	6.1
Phe	21.0	20.7	19.9
Glucosamine[c]	21.8	16.3	15.6
Galactosamine	430.9	343.6	247.6
Quantity (g)/(150 g of cartilage)	3.9	0.79	0.56

[a] Amino acid and amino sugar composition of a 48-hr cartilage extract (1) and two successive 24-hr extractions of the remaining insoluble tissue. [b] Residues per 1000 residues peptide. [c] Residues per 1000 total residues.

A sample (100 mg) of the major DEAE-Sephadex fraction was separated by ultrafiltration according to the scheme outlined in Figure 4. The amino acid analyses of the various fractions (Table V) suggest that glycine, glutamic acid, tyrosine, and lysine occur near substituted serines whereas the aliphatic residues (valine, isoleucine, leucine) are not present near these *loci*.

Based on the results of the ultrafiltration studies, an attempt was made to further resolve the major DEAE-Sephadex fraction by gel permeation chromatography on Sephadex G-100. Two poorly resolved fractions were obtained which were ultimately separated by rechromatography on the same column (Figure 5). The molecular weight distribution of these two fractions showed considerable overlap, heterogeneity and possible association. Since the fractions have essentially identical amino acid composition and, the chondroitin 4-sulfate chains are homogeneous as regards molecular weight it may be concluded that the observed size heterogeneity arises from differing degrees of saccharide chain substitution on the peptide cores or from variations in peptide length resulting in quantized molecular weights: peptide-(C-4-S)$_3$, peptide-(C-4-S)$_4$, peptide-(C-4-S)$_5$, etc. (Table VI).

The substitution stoichiometry of the peptide core was evaluated by the β-elimination–sulfite addition reaction. Samples of the Sephadex G-100 fractions and the main ultrafiltration fractions were treated with alkaline sulfite as previously described (Simpson and Davidson, 1972). Analytical data based on serine loss and cysteic acid formation indicate that about one-half (89 of 173 residues per 1000 residues peptide) of the serine residues were substituted with saccharide

FRACTIONATION OF PROTEOGLYCAN BY ULTRAFILTRATION

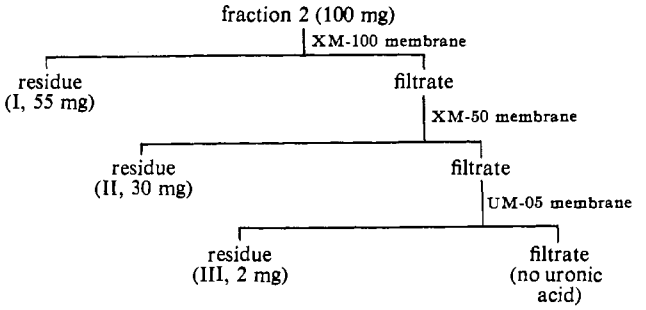

FIGURE 4: Scheme for fractionation of partially purified proteoglycan (fraction II, Figure 3) by ultrafiltration. The exclusion characteristics of the various Amicon membranes employed are given in footnote 3. Recovery of uronic acid and amino sugar was nearly quantitative. For analytical details, see Table V.

chains. The molecular weight of the chondroitin 4-sulfate chains based on the ratio of galactosamine present to cysteic acid formed was 14,000, a figure in good agreement with that determined independently by equilibrium sedimentation (M_w = 13,960; $M_z/M_w < 1.10$) following resolution of chains and peptide by ion-exchange chromatography.

DEAE-Sephadex fractionation of the second calcium chloride extraction produced a pattern similar to that of the first with a somewhat higher proportion of glucosamine enriched material, presumably representing keratan sulfate. A small fraction of this type was eluted prior to the main proteoglycan peak while a second such fraction emerged afterward. The composition of protein present in the initial eluate was characteristically similar to that of collagen (Table VII).

An evaluation of material prepared by guanidinium chloride extraction revealed essentially identical fractionation prop-

TABLE V: Amino Acid Analysis of Ultrafiltration Fractions.[a]

Amino Acid[b]	I	II	III
Lys	17.0	18.5	71.4
His	17.6	10.1	9.6
Arg	13.2	17.6	3.9
Asp	64.5	63.4	85.9
Thr	56.0	42.2	46.6
Ser	171.2	172.8	221.7
Glu	145.3	154.4	166.7
Pro	100.6	103.7	26.1
Gly	156.3	161.1	204.3
Ala	67.1	67.3	79.2
Val	51.2	52.3	21.1
Ile	35.8	42.1	17.5
Leu	75.5	72.9	27.8
Tyr	8.6	4.5	12.5
Phe	20.1	14.0	5.6
Glucosamine[c]	23.8	16.6	
Galactosamine	634.6	600.0	796.9

[a] Composition of fractions obtained by ultrafiltration of a DEAE-Sephadex fraction. The fractionation scheme is described in Figure 4. See text for details. [b] Residues per 1000 residues peptide. [c] Residues per 1000 total residues.

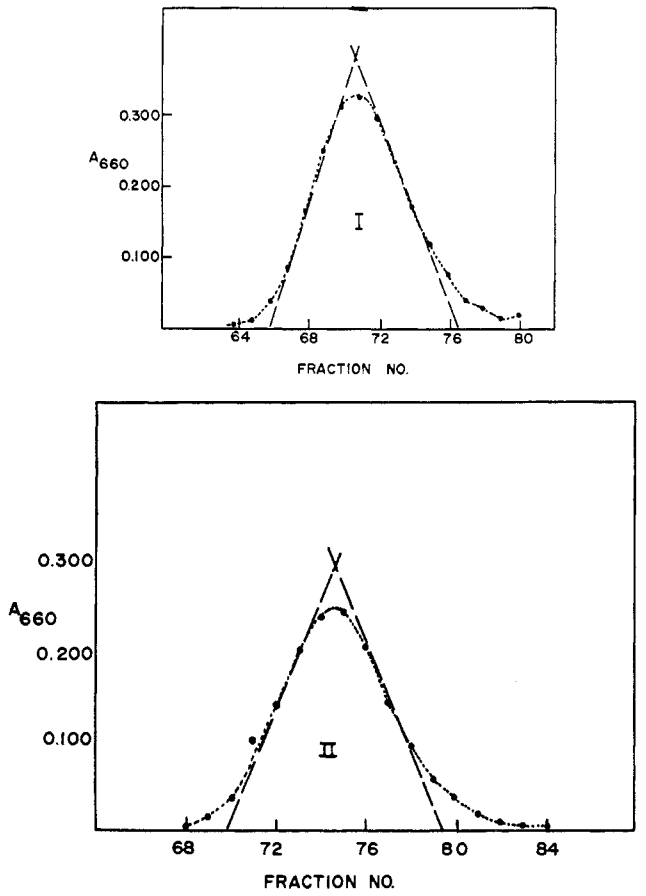

FIGURE 5: G-100 Sephadex rechromatography of fractions I (upper) and II (lower). Aliquots of each fraction were analyzed for uronic acid by the orcinol reaction. See text for details.

erties, amino acid analyses, and proteoglycan yields. Somewhat more collagen was solubilized by the guanidinium chloride but could be readily resolved from the proteoglycan. The molecular weight distributions were the same as those evaluated for the third calcium chloride experiment detailed above. Analytical (weight) comparison of proteoglycan prepared following dispersive extraction (Gerber *et al.*, 1960) and preliminary separation by ultracentrifugation showed no significant differences in amino acid or amino sugar composition. Somewhat lower molecular weights were observed probably reflecting increased proteolysis under the extraction conditions employed.

TABLE VI: Molecular Weight Parameters of Sephadex G-100 Fractions.[a]

	I	II
Sigma W	58,350–129,600	49,600–112,200
Sigma W (midpoint)	96,850	74,870
Sigma Y_1	37,550–90,800	21,000–63,300

[a] Molecular weights determined by sedimentation equilibrium. Partial specific volumes and a description of the computation procedures employed have been described (Woodward *et al.*, 1972). Sigma Y_1 is a computed molecular weight corrected for nonideality.

TABLE VII: Analysis of Nonretarded Protein.[a]

Amino Acid[b]	Protein	Collagen[c]
Lys	21.0	28.0
His	5.1	5.8
Arg	52.2	47.1
Asp	62.1	47.0
Thr	27.2	18.0
Ser	35.7	36.0
Glu	93.4	72.2
Pro	121.7	123.4
Gly	317.6	319.0
Ala	104.5	113.5
Val	21.2	23.6
Met	5.4	5.3
Ile	11.6	13.3
Leu	32.8	25.5
Tyr	6.1	4.5
Phe	16.0	13.9
Hyp	94.0	100.2
Glucosamine[d]	30.1	

[a] Composition of protein not retarded by DEAE-Sephadex chromatography of a cartilage extract. [b] Residues per 1000 residues peptide. [c] Eastoe (1967). [d] Residues per 1000 total residues.

Discussion

The dissociative extraction of cartilage with $CaCl_2$ solubilizes the bulk of the chondroitin sulfate containing proteoglycan while avoiding degradation arising from high-speed homogenization and excessive shear forces. Although some catheptic proteolysis probably takes place prior to extraction, the products isolated are presumed to be fairly representative of those present in the connective tissue structure. A successful reconstruction of all *in vivo* interactions is not possible at present but several chemical parameters of the proteoglycans and their behavior in the tissue can be defined.

The amount of proteoglycan remaining after three sequential dissociative extractions represents less than 5% of the total initially present based on residual amino sugar content. The material solubilized in the first 48-hr extraction period contains 80% of the chondroitin sulfate proteoglycan while successive fractions show increasing amounts of collagen and a slight increase in glucosamine content, possibly representative of keratan sulfate.

Chromatography of the crude proteoglycan preparation on DEAE-Sephadex in the presence of urea and salt, conditions necessary to minimize aggregation, resolve collagen-like protein and a glycoprotein component from the major uronic acid and galactosamine containing macromolecule which emerges from the column in a broad peak. The amino acid composition of the proteoglycan thus eluted is reasonably consistent throughout the peak and reproducibly free of sulfur containing amino acids. These results are in agreement with those obtained on analysis of the purified peptide core of the major cartilage proteoglycan isolated free of saccharide chains following elimination–sulfite addition (Simpson and Davidson, 1972). The small amount of cystine and methionine present in the crude extract is associated with material not retarded

on the DEAE-Sephadex column, a fraction free of galactosamine and uronic acid.

Further fractionation of the partially purified proteoglycan on QAE-Sephadex indicated that material appearing later in the salt gradient contained a higher proportion of keratan sulfate chains. Examination of the amino acid analyses of the QAE-Sephadex fractions shows significant changes in threonine and serine and minor alterations in some of the other amino acids. The otherwise very similar composition of the fractions suggests that a population of proteoglycans is present wherein the keratan sulfate chains are linked to threonine residues on the same peptide core that contains chondroitin sulfate chains, or that two general classes of proteoglycans are present wherein the keratan sulfate and chondroitin sulfate chains are linked exclusively to specific peptides of very similar but not identical composition. A possible explanation for the analytical values of the two QAE-Sephadex fractions may be that the presence of keratan sulfate chains on specific threonyl residues renders that region of the polypeptide core more resistant to proteolysis thus preserving those sections. Since threonine is not commonly located near seryl residues that have chondroitin sulfate chains attached, it may be argued that if keratan sulfate chains are on the same polypeptide core, they are relatively distant from the CS chains. In any case, the fractionation properties of the proteoglycan are largely determined by their net charge since size heterogeneity seems omnipresent. Thus, clear resolution of keratan sulfate containing molecules would not be expected unless significant oversulfation were present. The possibility of a specific peptide bearing only keratan sulfate chains remains and is not excluded by the analytical data presented.

Similar conclusions can be drawn from an examination of the results of the preparative disc gel electrophoresis experiments. The first peak eluted was free of glucosamine, contained about 3% protein on a weight basis, and had a molecular weight comparable to that of free chondroitin sulfate chains. The amino acid analysis showed that glycine, lysine, tyrosine, and possibly glutamic acid were enriched compared to the starting material suggesting that these residues are frequent near substituted serine *loci*. In contrast, the threonine content of this fraction is much lower compared to the starting material whereas it is enriched in the fraction containing the bulk of the glucosamine. Examination of the various proteoglycan fractions on analytical acrylamide gels revealed no significant mobility differences indicating that for those samples able to enter the gel matrix, mobility is largely, if not exclusively, determined by their respective charge densities.

An attempt to further purify proteoglycan by selective ultrafiltration showed that in spite of significant size heterogeneity, there was no difference in composition between the major components. A small amount of nearly peptide-free chondroitin sulfate chains were isolated which had residual peptide of comparable amino acid composition to that of material isolated following preparative disc gel electrophoresis. Once again, glycine, lysine, tyrosine, and glutamic acid were enriched whereas the aliphatic amino acids valine, isoleucine, and leucine as well as threonine were markedly lower. These data strongly indicate the nature of the residues near those serines which are substituted by chondroitin sulfate chains.

Based on the ultrafiltration studies, an attempt was made to resolve proteoglycans of different size by gel permeation chromatography. The molecular weight distribution of the isolated fractions showed heterogeneity and considerable overlap. In view of the fact that the saccharide chains are homogenous in size (14,000), it seems reasonable to conclude

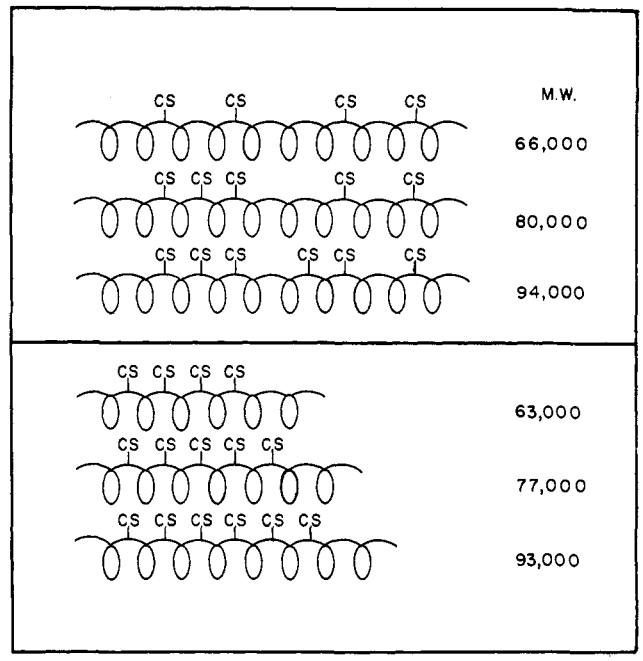

FIGURE 6: Schematic drawing of possible components of the proteoglycan fractions studied. The upper set represents a peptide of fixed size to which varying numbers of chondroitin sulfate (CS) chains are attached. The lower set represents peptides of increasing size to which progressively greater numbers of CS chains are attached. The approximate molecular weights for each representative are indicated.

that the observed variation in molecular weights arises from different degrees of saccharide substitution on the peptide core or from a variation in peptide size which leads to fragments containing different numbers of chains. The resolution of this problem requires a detailed sequence study of the core peptide, currently in progress.[4] A schematic illustrating these possibilities is presented in Figure 6.

The substitution stoichiometry of the major proteoglycan fractions isolated after DEAE-Sephadex chromatography and ultrafiltration showed that about half of the serines present was substituted. The molecular weight of the saccharide chains was calculated from the ratio of galactosamine to cysteic acid and was in excellent agreement with that measured by equilibrium sedimentation.

The macromolecular properties of the proteoglycan apparently involve associative behavior which may involve counterions and other proteins as well as the aggregating tendency of the proteoglycan itself. An extrapolation of the observed chemical profile to the native or biosynthetically formed covalent structure seems premature at present.

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Binding of Adenosine Triphosphate to Myofibrils during Contraction and Relaxation†

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ABSTRACT: It is possible to measure ATP binding to myofibrils under conditions of rapid hydrolysis, because free ATP can be maintained phosphorylated with the creatine phosphate–kinase system. Nevertheless, it is very likely that not all of the nucleotide is ATP but that some or most of it is ADP bound to the three or possibly four nucleotide-containing enzyme–substrate and enzyme–substrate–cofactor intermediates that must exist. Nucleotide binding to myofibrils can be described fairly accurately by two reciprocal K_M values, one for 25% and the second for the remainder of the sites. Removal of calcium, while apparently not affecting the first, increased the second $1/K_M$ about fivefold. The total number of binding sites was equal to the total number of myosin "heads" and was the same in the presence and absence of calcium. This latter observation is not compatible with the concept that during relaxation ATP is bound to a special "relaxing" site from which it is displaced in the presence of calcium. Arguments are presented in favor of explaining the change of K_M of ATP binding on calcium removal by the reduction of the rate of ATP hydrolysis through inhibition of the reaction actin + myosin ~ → A~M (force-generating complexes). The difference in the K_M of 25% of the total sites may be explained in a number of ways without it being possible at this time to decide which one is correct. Among the possibilities is a form of negative cooperativity that differs from the usual mechanism and was therefore modeled.

Muscle requires ATP not only for contraction but also for relaxation and the maintenance of the resting state. After removal of ATP, most of the actin and myosin molecules form bonds with each other, causing the muscle to go into rigor. By contrast, during rest, muscle is very extensible because actin and myosin filaments are completely dissociated from each other. ATP is needed to break the complex between actin and myosin—an action for which polyphosphates, such as nucleoside di- and triphosphates and inorganic pyrophosphate seem to be specific. However, ATP causes dissociation of the actomyosin complex not only during relaxation but also during shortening. The data by Lymn and Taylor (1971) indicate that dissociation follows immediately the binding of ATP to actomyosin. The dissociated state is transitory during contraction: it is terminated by the formation of force-generating links between activated myosin and actin. During relaxation dissociation is permanent, because this recombination of actin and myosin is prevented by calcium-free troponin (Ebashi and Ebashi, 1964; Ebashi *et al.*, 1968). However, troponin can prevent the formation of active actin–myosin links only if sufficient ATP is present. At low ATP, in spite of the removal of calcium, tension is developed (Reuben *et al.*, 1971; White, 1970), shortening and syneresis take place (Weber and Herz, 1963; Levy and Ryan, 1965; Endo, 1964), and ATP hydrolysis is activated by actin (Weber and Herz, 1963; Weber, 1969). It appears then, that ATP is not only responsible for the breaking of the complex between the two proteins, but, in addition, in some way is involved in the reaction by which calcium-free troponin prevents the formation of an active actin–myosin complex. We wondered (Weber *et al.*, 1964) as did others (Levy and Ryan, 1965) whether ATP may bind to a relaxing site, in addition to the hydrolytic site. Therefore, we wanted to know the total number of binding sites for ATP in the myofibril, and find out whether under relaxing conditions (in the absence of calcium and at high ATP) more ATP is bound than in the presence of calcium.

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