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The Subunits of Chemically Treated Proteoglycan Isolated from Bovine Nasal Cartilage

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1. The light fraction of the proteoglycan of bovine nasal cartilage was split by treatment with 0.1M-hydrochloric acid in acetone. The products were separated by gel filtration on 4% agarose and two retarded fractions were detected and isolated. These two fractions were found to have a Stokes radius of 134 and 47 Å respectively, as determined by calibration of the column against proteins of known hydrodynamic volumes. 2. The 47 Å fraction had a protein content of 4% and a glucosamine/galactosamine ratio 1:23. The 134 Å fraction had a protein content of 20% and a glucosamine/galactosamine ratio 1:4.8. 3. The results of the viscometric studies on both fractions suggested that the 134 Å fraction alone exhibited the property of undergoing reversible pH-dependent aggregation with a transition point at pH 4.9. 4. It was concluded that these fractions could represent subunits of the native cartilage proteoglycan.

Serafini-Fracassini (1968) reported that the core of the proteoglycan isolated from bovine nasal cartilage can be cleaved by dry 0.1M-hydrochloric acid in acetone, under mild experimental conditions. Approximately 90% of the acetone-treated material was precipitated by stepwise alcohol fractionation over a very narrow range of ethanol concentration. Results of chemical analyses and of molecular-weight determinations carried out on this fraction and on the original untreated material indicated breakdown of the macromolecular organization of the proteoglycan without apparent damage to the protein core and the glycosaminoglycan chains. The ultracentrifugal analysis of the main fraction of the treated proteoglycan showed a bimodal pattern with peaks characterized by sedimentation coefficients of 8.1S and 2.3S respectively.

The object of the present investigation was to isolate these two macromolecular species and to determine their physicochemical parameters and compositions.

A preliminary report of some of these results has appeared (Serafini-Fracassini & Stimson, 1970).

MATERIALS AND METHODS

Isolation of cartilage proteoglycan. Chondromucoprotein was extracted from fresh bovine nasal septa by the procedure of Malawista & Schubert (1958), by using low-speed homogenization to prevent mechanical depolymerization and cooling the suspension in a low-temperature bath to prevent the temperature rising above 4°C. The extract was fractionated by high-speed centrifugation as described by Gerber, Franklin & Schubert (1960). The proteoglycan was precipitated from the supernatant by addition of 2 vol. of ethanol, redissolved in 0.5M-KCl and centrifuged at $78000g_{av.}$ for 1h. All manipulations were carried out as quickly as possible at 4°C to avoid proteolytic degradation. After alcohol precipitation, the protein–polysaccharide complex was dissolved in 30% (v/v) ethanol and an aqueous saturated solution of $CaCl_2$ was slowly added while the solution was vigorously stirred. When a strong turbidity developed, 1 vol. of ethanol was added and the precipitate was collected by centrifugation, washed with ethanol, dried and stored under reduced pressure over P_2O_5. The average yield was approx. 23% of cartilage dry weight. The composition of this material was: protein 17.5%, hexosamine 26.2% and hexuronic acid 29.0%.

Acid–acetone treatment and alcohol fractionation. The proteoglycan was treated with freshly prepared dry 0.1M-HCl in acetone for 1h at room temperature as described by Serafini-Fracassini (1968). The product was subsequently washed with acetone and dried.

The acetone-treated material was dissolved in water and the pH of the solution was adjusted to 7. Ethanol was then slowly added to give a final concentration of 75% (v/v) while the solution was vigorously stirred. The precipitate that formed over 12h was removed by centrifugation at $8000g_{av.}$ for 30min and discarded. The ethanol concentration of the supernatant was increased to 85% (v/v) and the precipitated fraction was recovered by filtration on a Teflon filter and dried in a desiccator over P_2O_5. The average yield was 92% of the untreated proteoglycan.

Analytical procedures. Hydrolyses for amino acid analyses were carried out under N_2 in sealed tubes with constant-boiling HCl (2ml/mg of material) at 110°C for 24h. Acid was removed from the hydrolysates in a rotary-film evaporator at 30°C. For subsequent analysis, a Technicon amino acid analyser was used. Modifications of

the buffer system employed have been discussed by Serafini-Fracassini, Peters & Floreani (1967).

Amino end-group analysis was carried out by the 2-chloro-3,5-dinitropyridine method (Signor, Biondi, Terbojevich & Pajetta, 1964), as described by Serafini-Fracassini (1968). The dinitropyridyl-substituted material was hydrolysed with constant-boiling HCl in sealed tubes, under N_2, at 60°C for 10h. The hydrolysate, once brought to pH2 with KOH, was extracted with six portions (10ml each) of ethyl acetate. The pooled ethyl acetate extracts were washed twice with 5ml of 0.01M-HCl. The water phase together with the acid washings of the ethyl acetate extracts were evaporated to dryness and the residue was dissolved in constant-boiling HCl and hydrolysed at 110°C for 24h before amino acid analysis. Quantitative determination of the *N*-terminal amino acids was carried out by comparing the amino acid concentrations of this hydrolysate with those of the starting material.

For hexosamines, samples of known weights (approx. 1mg) were dissolved in 2ml of 4.5M-HCl and hydrolysed in sealed tubes, under N_2, at 105°C for 8h. After hydrolysis, excess of acid was neutralized and total hexosamine was determined by the Elson & Morgan (1933) reaction by using the distillation procedure of Cessi & Piliego (1960). Differential determination of glucosamine and galactosamine was carried out by column chromatography (Partridge & Elsden, 1961).

Hexuronic acid was measured directly on unhydrolysed solutions by the method of Bitter & Muir (1962), with glucuronic acid as the standard.

The protein concentration of eluates from gel filtration was determined by the micro-biuret method of Itzhaki & Gill (1964).

Gel filtration. A column (1.5cm×90cm) was packed, as described by Flodin (1962), with agarose gel obtained from a commercial preparation (Bio-Gel A-15m, 100–200 mesh; Bio-Rad Laboratories, Richmond, Calif., U.S.A.). Before use the packed column was eluted with 1M-potassium acetate, adjusted to pH7 with acetic acid, under the conditions of flow rate required for subsequent experiments (9.5ml/h). The final bed volume was 124ml. The void volume was determined with narcissus mosaic virus and the gel column was calibrated by determination of the elution volumes of a series of proteins of known Stokes radius. These included bovine thyroglobulin (Edelhoch, 1960), glutamate dehydrogenase (Rogers, Hellerman & Thompson, 1965), yeast alcohol dehydrogenase (Hayes & Velick, 1954) and horseradish peroxidase (Cecil & Ogston, 1951). Samples of these standards (5mg each), dissolved in 1M-potassium acetate, pH7, were applied to the column in 1.5ml portions for zonal analysis. Fractions (1.5ml each) were collected, the effluent was continuously monitored at 260nm with a base-compensating automatic recorder, and each fraction was tested for its protein content.

A second column (2.5cm×60cm) was packed with 1% agarose (Bio-Gel A-150m, 100–200 mesh; Bio-Rad Laboratories) and equilibrated with 1M-potassium acetate, pH7, as described above. The flow rate was adjusted to 12ml/h.

Proteoglycan preparations (20mg each), dissolved in 1M-potassium acetate, were applied to the columns in 1.5ml samples. Effluent fractions were monitored by determination of their hexuronic acid content.

Viscometry. Viscosity measurements were made at 25±0.01°C with a capillary viscometer constructed as described by Fox, Fox & Flory (1951). The rate of shear characteristic of this instrument, when filled with 7ml of water, was $551\,s^{-1}$. Samples of fractions 1 and 2 (see the Results section) were dissolved in 0.05M-KCl at a concentration of approx. 0.2g/100ml. The pH was then adjusted in the range 2.5–7.5 and 7ml of each solution was pressure-filtered into the viscometer through no. 2 sintered glass.

Osmometry. The instrument used was a model 501 high-speed osmometer (Hewlett–Packard, Avondale, Pa., U.S.A.) equipped with B-19 membranes (Schleicher and Schuell, Keene, N.H., U.S.A.). The constant-temperature control was set at 20°C. All osmotic-pressure measurements were made with a high concentration of diffusible electrolyte. Each sample was dissolved in and dialysed against 2M-KCl, adjusted to suitable pH with HCl. Dialyses were carried out for 4 days at 4°C. Proteoglycan concentrations were determined before and after dialysis by determination of the hexuronic acid content of the solutions and by differential refractometry. Progressive dilutions of each sample were prepared and at least four osmotic-pressure determinations were made for each concentration.

Electron microscopy. An aqueous solution of acetone-treated proteoglycan was sprayed at a concentration of approx. 3μg/ml on to carbon-coated grids. Once dry, the grids were stained for 10min in 0.5% (w/v) bismuth nitrate in 0.1M-HNO_3 (Serafini-Fracassini, Durward & Crawford, 1969). They were then washed in 0.1M-HNO_3 followed by water and dried under reduced pressure in a desiccator. Micrographs were taken with an AEI EM6B electron microscope.

RESULTS

The gel-filtration results obtained for the four proteins chromatographed for the calibration of the 4% agarose column were used in the elaboration of the plot of $(-\log K_{av.})^{\frac{1}{2}}$ versus Stokes radius, as described by Laurent & Killander (1964), as shown in Fig. 1. The high ionic strength of the buffer used in the elution of the gel column was intended to avoid adsorption phenomena and particularly to decrease the repulsive effects among fixed charges on the glycosaminoglycan chains to maximize the uniformity of their effective hydrodynamic volumes in a random-coil conformation. The elution profile of the acetone-treated proteoglycan on the 4% agarose column is shown in Fig. 2. The retarded material, accounting for approx. 55% of the recovered hexuronic acid, was resolved into two fractions with elution volumes of 70ml (fraction 1) and 109ml (fraction 2). These values were converted, on the basis of the calibration curve shown in Fig. 1, into the corresponding Stokes radii of 134 and 47Å respectively.

To remove high-molecular-weight species contaminating fraction 1, as indicated by analytical ultracentrifugation that revealed a rapidly sedimenting material (Serafini-Fracassini, 1968), a

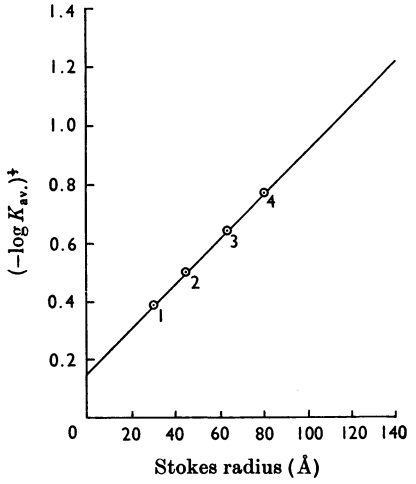


Fig. 1. Plot correlating the elution volume and the Stokes radius of four proteins on a 4% agarose column (bed volume 124ml, void volume 52ml) at pH7. Experimental details are described in the text. 1, Bovine thyroglobulin; 2, glutamate dehydrogenase; 3, yeast alcohol dehydrogenase; 4, horseradish peroxidase. $K_{av.}=(V_e-V_0)/(V_t-V_0)$, where V_e=elution volume of solute under investigation. V_0=void volume of the column, V_t=total volume of the gel bed.

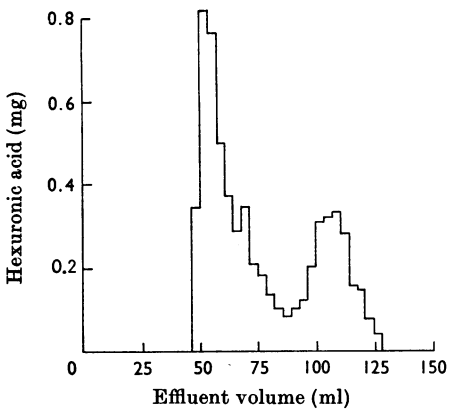


Fig. 2. Elution of acid–acetone-treated proteoglycan (expressed as hexuronic acid) with 1M-potassium acetate, pH7, from a column of 4% agarose (bed volume 124ml, void volume 52ml).

preliminary separation was carried out on the 1% agarose column and a bimodal elution pattern was obtained (Fig. 3). The whole of the second peak (at 28–55ml) was rechromatographed on 4% agarose and a pattern similar to that reported in Fig. 2 was obtained, although the excluded material was much decreased.

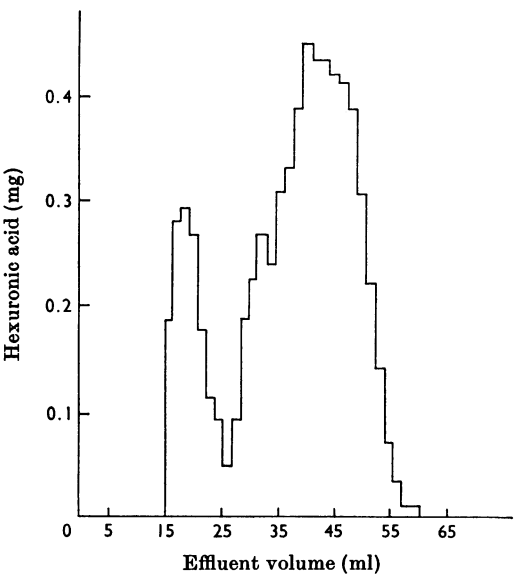


Fig. 3. Elution of acid–acetone-treated proteoglycan (expressed as hexuronic acid) with 1M-potassium acetate, pH7, from a column of 1% agarose. Void volume 18ml.

Fraction 1 (at 65–85ml) and fraction 2 (at 95–120 ml) were isolated from the eluates of a series of chromatographic separations on 4% agarose after prior purification on 1% agarose.

The viscosities of the two fractions were determined at different H^+ concentrations. Fig. 4 shows plots of reduced viscosity versus pH. Potassium chloride at a concentration of 0.05M was used as the solvent, this being an ionic concentration at which the viscosity of the proteoglycan has been shown to be independent of the amount of salt present in solution (Luscombe & Phelps, 1967). Whereas fraction 2 gave a normal curve over the pH range examined, fraction 1 showed an anomalous curve with a major inflexion point at pH4.9.

Concurrently, molecular-weight determinations were carried out by osmometry on both fractions at pH7. An additional molecular-weight determination of fraction 1 was performed at pH3.5 as the H^+ concentration appeared to affect the physicochemical parameters of this macromolecular system in solution. The plots of the reduced osmotic pressures (Π/c) as functions of solute concentrations (c) (Fig. 5) are consistent with a number-average molecular weight of 750000 for fraction 1 at pH7 and 122500 for the same fraction at pH3.5. A molecular weight of 47400 was calculated for fraction 2 (Fig. 6).

Plate 1 shows a typical field of the monolayer produced by spraying the acetone-treated proteoglycan on carbon-coated grids. Together with long

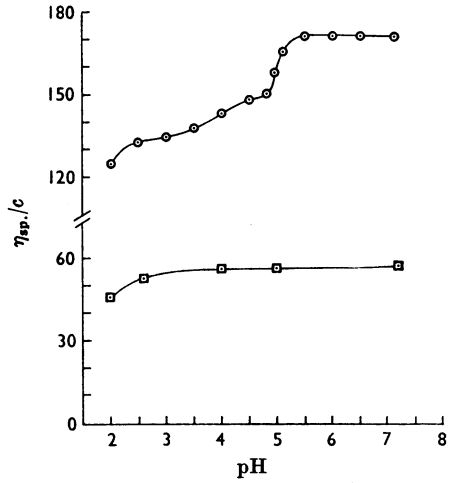


Fig. 4. Plots of viscosity number versus pH for fraction 1 (⊙) and fraction 2 (⊡) at a glycosaminoglycan concentration of 0.2% in 0.05M-KCl, at 25°C and 551 s^{-1} shear rate.

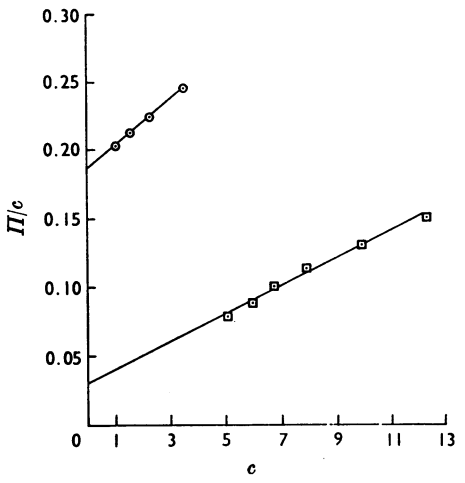


Fig. 5. Plots of Π/c versus c obtained for fraction 1 in 2M-KCl at pH 3.5 (⊙) and pH 7 (⊡).

beaded filaments (arrows *a*), interpreted as polymeric forms, both short segments, composed of three 30 Å particles (arrows *b*), and isolated particles (arrows *c*) can be seen.

The amino acid compositions of fraction 1 and fraction 2 are reported in Table 1. Each amino acid concentration was corrected for hydrolytic losses by applying the coefficients reported by Serafini-Fracassini *et al.* (1967). The protein contents, obtained by summation of these corrected values, are reported in Table 2 together with the results of all other chemical analyses.

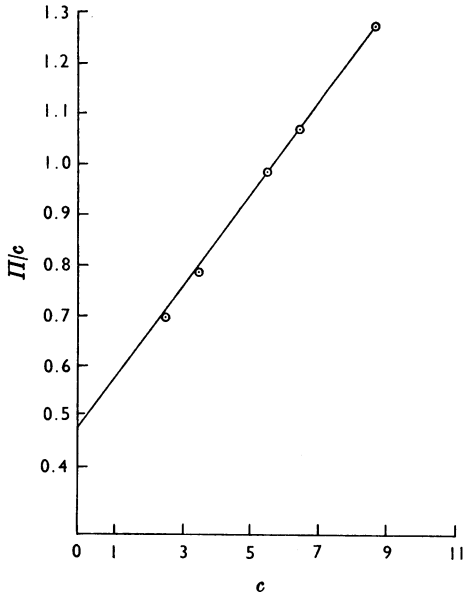


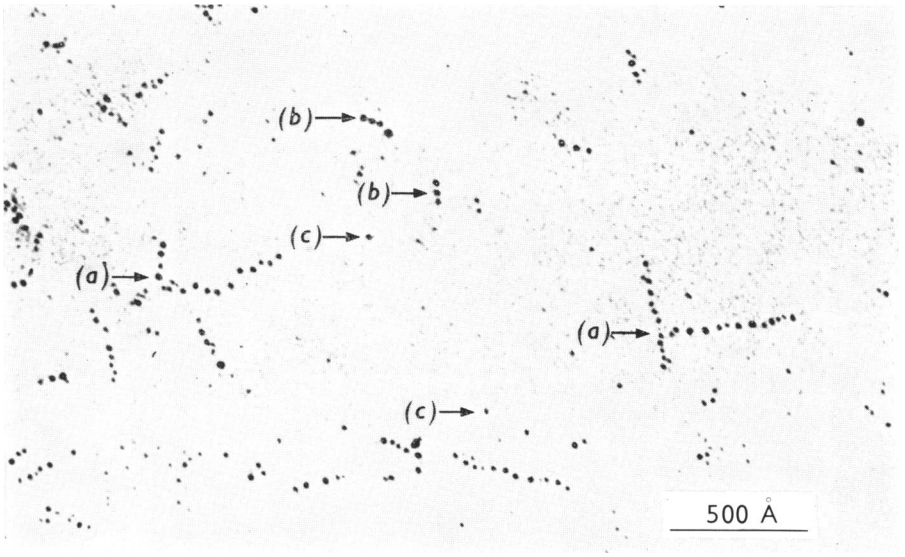
Fig. 6. Extrapolation of the reduced osmotic pressure of fraction 2 in 2M-KCl, pH 7.

Table 1. *Amino acid analyses*

Values are expressed as μg of anhydro-amino acid/10 mg of ash- and moisture-free samples.

Sample ...	Fraction 1	Fraction 2
Amino acid		
Hyp	0.0	0.0
Asp	226.1	37.4
Thr	110.1	23.0
Ser	154.2	39.3
Glu	343.4	71.8
Pro	139.5	37.7
Gly	124.0	38.3
Ala	91.0	23.0
Val	129.5	12.8
Cys	0.0	0.0
Met	0.0	0.0
Ile	77.1	12.1
Leu	179.5	29.0
Tyr	56.8	11.4
Phe	115.1	18.2
Hyl	0.0	0.0
Lys	76.3	11.3
His	37.1	0.0
Arg	140.3	26.7
Total	2000.0	392.0

The results of the amino end-group determination of fraction 2 indicated the presence of two *N*-terminal amino acids. These were leucine and isoleucine at concentrations of 15.8 and 9.0 mol/10^6 g respectively. The molecular weight calculated from the total number of mol of *N*-terminal amino acids/



EXPLANATION OF PLATE 1

Monolayer of acid–acetone-treated proteoglycan sprayed on to a carbon-coated grid at a concentration of 3 μg/ml. The bismuth nitrate staining technique was used. Arrows (*a*) indicate long beaded filaments composed of several 30 Å particles. Arrows (*b*) point to short beaded segments composed of three 30 Å particles and arrows (*c*) point to isolated particles.

Table 2. *Chemical analyses*

Values are expressed as percentages of dry ash-free samples.

Sample ...	Fraction 1	Fraction 2
Protein	20.0	3.9
Total hexosamine (as free base)*	23.8	32.1
Glucosamine/galactosamine ratio	1:4.8	1:23.1

* These values are not corrected for hydrolytic losses.

10^6g of proteoglycan is 40300, which is in keeping with the value obtained by osmometry on the same fraction.

DISCUSSION

The combined information gathered from analytical gel filtration and ultracentrifugation (Serafini-Fracassini, 1968) indicates that the acetone-treated proteoglycan preparation examined is composed of a high-molecular-weight fraction and of two discrete macromolecular species.

The smaller of these two macromolecular species, which behaves as a single homogeneous fraction (fraction 2) during gel filtration on 4% agarose and which corresponds to the fragment characterized by a sedimentation coefficient of 2.3S, has a number-average molecular weight 47400. Its effective hydrodynamic volume is expressed by a Stokes radius of 47 Å in 1 M-potassium acetate. The other macromolecular fragment, which is eluted during gel filtration on 4% agarose as fraction 1, exhibits a Stokes radius of 134 Å and molecular weight 122500 at pH 3.5. These physicochemical parameters would suggest that the latter macromolecules are constituted of three molecules similar in size to the former units and assembled together by an end-to-end alignment of their core polypeptides.

It is noteworthy, however, that the compositions of the two macromolecular species reveal large differences. Fraction 2 has a protein content of 4% and a very low glucosamine concentration, which is probably attributable to a contamination of keratan sulphate. Fraction 1, on the other hand, has a much higher protein content (20%) and a glucosamine/galactosamine ratio of 1:4.8. Serafini-Fracassini (1968) reported that the number-average molecular weight of the chondroitin sulphate chain of a similar acetone-treated proteoglycan preparation was determined by osmometry, after cleavage of a proportion of the protein moiety by *N*-bromosuccinimide, to be approx. 19000. Therefore fraction 2 appears to be constituted by two chondroitin sulphate chains linked by a short polypeptide. When the amino acid concentrations of this polypeptide are expressed as number of amino acid residues per 47000 molecular weight unit, 2.1 residues of serine are found to be present and presumably engaged in the glycosidic linkage with the glycosaminoglycan chains. Four *N*-terminal amino acids were previously detected in the acetone-treated proteoglycan (Serafini-Fracassini, 1968). These were also found to be present in the untreated proteoglycan (Serafini-Fracassini *et al.* 1967), thus precluding the possibility that they might have arisen through cleavage of peptide bonds, in the proteoglycan core, that are particularly labile to acid conditions. Of these four amino end-groups only two are present in fraction 2, indicating that at least two different core polypeptides are associated with chondroitin sulphate in bovine nasal cartilage.

If it is assumed that all glucosamine were derived from keratan sulphate, with a chain weight of approx. 10000 (Mathews & Cifonelli, 1965), the molecular weight of fraction 1 and its glucosamine content would indicate the presence of four chondroitin sulphate and two keratan sulphate chains in the same macromolecular unit. The high protein content of fraction 1 could then be attributable to the keratan sulphate moiety, this being in keeping with the findings of Hoffman, Mashburn, Meyer & Bray (1967) and Hoffman, Mashburn & Meyer (1967).

Although the presence of keratan sulphate does not prevent the core of the proteoglycan from being split by the acid–acetone treatment, it seems to stabilize the structure, in which it is involved, to the extent of avoiding its complete degradation to fragments similar in size to those present in fraction 2.

It is noteworthy that fraction 1 has a molecular weight of 750000 as determined by osmometry under conditions similar to those used in gel filtration. Such a value is not in agreement with that which is expected considering the Stokes radius of the macromolecule, if a comparison between fractions 1 and 2 can be drawn. However, when osmometric determinations are carried out at pH 3.5, the values for molecular weight and hydrodynamic volume are consistent. This can be interpreted as being due to the ability of the 134 Å species to undergo aggregation at neutral pH in spite of the high ionic strength of the buffer used in both experiments. If this hypothesis is correct, fraction 1, as shown in Fig. 2, is constituted by isolated molecules in equilibrium with aggregates excluded by the agarose gel. The isolation of fraction 1 appears to alter this equilibrium, thus resulting in aggregation of free 134 Å molecules until a new equilibrium is attained, unless the process is prevented by lowering the pH.

The results of the viscosity experiments carried

out on fraction 1 are in agreement with this view and suggest that the aggregation is reversed as the result of a change in the H^+ concentration of the system, with a fairly sharp transition point between association and dissociation at pH 4.9. The increase in asymmetry of the macromolecules, revealed by the rise in viscosity of the solution, could be interpreted as being due to an end-to-end association of several 134 Å molecules. A similar effect of solvent pH on macromolecular aggregation has been reported by Hascall & Sajdera (1969) in their study of a proteoglycan fraction, characterized by a sedimentation coefficient of approx. 20S, isolated from bovine nasal cartilage. These authors demonstrated that aggregation of the 20S subunits is initiated by non-covalent interaction between proteoglycan macromolecules and a specific glycoprotein. It is noteworthy that such a phenomenon is occurring both in systems composed of high-molecular-weight complexes and in solutions of low-molecular-weight proteoglycans, as shown in the present study. The sharp inflexion point, at pH 4.9, in the viscosity curve of fraction 1 indicates that probably complete ionization of the carboxyl groups is necessary for aggregation to occur.

The existence of the 134 Å species as a macromolecular entity is further supported by the electron micrograph, shown in Plate 1, in which short segments composed of three electron-dense particles are evident. In previous papers (Serafini-Fracassini & Smith, 1966; Smith, Peters & Serafini-Fracassini, 1967; Serafini-Fracassini *et al.* 1969) the specificity of Bi^{3+} for glycosaminoglycans, under the experimental conditions used during the staining of the grids, has been proved and a correlation between each 30 Å particle and a doublet of polysaccharide chains has been made. Moreover, in this electron micrograph the 60 Å period along the segments is still present which is in keeping with earlier observations on the native proteoglycan (Serafini-Fracassini & Smith, 1966) and with the Mathews & Lozaityte (1958) model for chondromucoprotein.

The isolation of two distinct macromolecular species after acid–acetone treatment of cartilage proteoglycan implies the existence of labile bonds periodically distributed along the core of the native complex. These are not envisaged as peptide bonds. Although the material examined underwent chemical treatment and it is possible that the 47 Å species and 134 Å species arose from different molecules, this study suggests that these species may represent true subunits of the protein–polysaccharide complex of bovine cartilage. If this hypothesis is correct, the proteoglycan could be considered to be constituted of a family of closely related macromolecules differing only in the relative amounts of the various component subunits.

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