Isolation and Physical Characterization of Hyaluronic Acid Prepared from Bovine Nasal Septum by Cetylpyridinium Chloride Precipitation*

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Raw extract in 2 M CaCl₂ of bovine nasal septum cartilage was eluted from 4% agarose gel to give a "void volume" Fraction v-4, which was indistinguishable in composition and behavior on viscometric and sedimentation analysis from the densest fraction obtained by associative centrifugation in a cesium chloride density gradient. The sulfated proteoglycan was precipitated (Fraction A) by cetylpyridinium chloride from acidic solutions of Fraction v-4 or of dialyzed raw extract. Neutralization under conditions of low ionic strength precipitated a further small fraction (B), which contained from 0.5 to 1% of the uronic acid in the original extract. Analysis by associative and dissociative density gradient centrifugation demonstrated that Fraction B resembled in effective density known samples of hyaluronic acid from other sources. Gel chromatography of proteolytic digests of Fractions A and B on 6% agarose indicated that cetylpyridinium chloride precipitation essentially separated sulfated proteoglycan (A) from hyaluronic acid (B). A viscosity-average molecular weight of about 5 \times 10^6 was estimated for a sample of Fraction B purified in a dissociative (GdnHCl-CsCl) density gradient. Sedimentation velocity data were consistent with this result. Analysis of hexosamines showed that the sample contained 96% glucosamine, confirming the identification of hyaluronic acid. The proteoglycan fraction (A) resembled "subunits" in its sedimentation behavior.

EXPERIMENTAL PROCEDURES

Materials—All substances were of reagent grade and were used without further treatment with the following exceptions. Reagent grade calcium chloride (CaCl₂•2H₂O) obtained from Fisher, was made to 2 M and filtered through a medium sintered glass funnel prior to use. Cetylpyridinium chloride and guanidine hydrochloride (Grade I) were obtained from Sigma. Cesium chloride was the product of 99% purity of Ventron Corp.; solutions of CsCl were centrifuged at 20,000 rpm (Beckman 50 rotor) for 15 min to remove insoluble material. Crude papain was obtained from General Biochemicals. The sources of agarose gel were: 2% gel, Sepharose 2B (Pharmacia), 4% gel, Bio-Gel A-15m (Bio-Rad); 6% gel, Sepharose 6B (Pharmacia). Unfractionated hyaluronic acid from rooster comb (RCH) has been described previously (7). Chondroitin 4-sulfate was used as the fraction eluted at 0.35 M NaCl from a large scale fractionation of a crude sample of chondroitin sulfate obtained from General Biochemicals. The fractionation procedure involved stepwise elution in NaCl solutions from the crude sample adsorbed on DEAE-cellulose, and resembled that used for hyaluronic acid (9) except that the elution range was 0.20 to 0.60 M NaCl with steps of 0.05 M. Keratan sulfate was one of the acid mucopolysaccharide reference standards obtained from Dr. J. A. Cifonelli, Department of Pediatrics, University of Chicago.

Cartilage tissue was prepared and extracted according to the method of Sajdera and Hascall (10). Nasal septa from cattle were obtained from the abattoir within 2 h of slaughter, cleaned of noncartilaginous tissue, sliced with a woodworking tool (Stanley Surform) and extracted overnight at 4° with 2 M CaCl₂ (3 ml/g of wet cartilage). Raw extract was freed of insoluble tissue by centrifugation at

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The abbreviations used are: GdnHCl, guanidine hydrochloride. Designations of particular samples of hyaluronic acid are made to correspond, where possible, to those originally used for these samples: RCH, rooster comb hyaluronic acid (7); UFR, ultrafilter residue from ox synovial fluid hyaluronic acid (8). Operative abbreviations for samples and fractions obtained from cartilage extracts are inspired by the system used by Hascall and Heinegard (3). Sample E, polymeric solutes in raw extract in 2 M CaCl₂ or the solution containing them; v- (or v+), the "void volume" fraction from a gel column, especially v-4, such a fraction obtained from Sample E on a 4% agarose column; -D, a fraction purified by centrifugation in a dissociative density gradient, especially D1, the first (denses) fraction; Al, the first (densest) fraction from centrifugation of raw extract on an associative (CsCl) density gradient; -P, an enzymatic digest with papain. Fractions A, B, and C (sometimes also indicating source, as v-4-A, E-A, etc.) refer to A, the precipitate obtained with cetylpyridinium chloride in dilute HCl solution; B, the precipitate obtained from the supernatant of A upon neutralization with Tris-C, the solute in the supernatant solution from the precipitation of B. Fraction BC refers to unseparated Fractions B and C.
Hyaluronic Acid of Cartilage

20,000 rpm (Beckman SW 25.1 rotor) for 15 min. The supernatant solution (or its polymeric content) was referred to as Sample E.

Analytical Methods — Uronic acid was determined by a modification (9) of the carbazole method of Dische (11). When hyaluronic acid was determined as the sodium salt, its concentration was calculated by dividing the uranic acid concentration by 0.45 (12). Protein was determined by the biuret method (9). Sodium ion at 0.2 M concentration. Fluorometric detection of primary amino acids was performed on a single column, high pressure analyzer with a microbore column, 0.32 cm in diameter (Durrum Chemical Corp., Palo Alto, Calif.), filled with Durrum DC-4A cation exchange resin. Stepwise elution was carried out with a series of three citrate buffers (pH 3.25, 4.15, and 5.25), all containing sodium chloride as observed by ultraviolet absorption at 260 nm. Amino acid analyses were performed on a Beckman model D amino acid analyzer described above with a 0.065 ml sample of a 2 h hydrolysate. Analysis was performed with the reagents specified by Helbert and Brown (18) with omission of their heating step, which was followed by centrifugation and decanting. A small amount of solid CsCl was applied to a column (0.9 x 12 cm). A sample of 50 to 60 ml of Sample E containing about 3 mg/ml of uranic acid, was weighed and assayed to determine its uranic acid content m in milligrams. To a sample volume V(ml) was added V, = m/66 + 0.05 V ml of 1 M HCl and 0.1 ml of 5% (v/v) cetylpyridinium chloride. The sample was weighed, and, assayed for uranic acid and protein. Fractions with a protein to uranic acid weight ratio less than 0.35 were pooled, dialyzed into 0.2 M NaCl at 5°, concentrated to 20-fold by rotary evaporation, and redialyzed into 0.2 M NaCl. The collected fraction was termed Fraction v-4-A.  

Preparation of Fraction v-4-A — Fraction v-4 was dialyzed into large excess of 0.02 M NaCl for at least 2 days at 4° with change of solvent twice daily. The dialyzed sample, typically containing about 1 mg/ml of uranic acid, was weighed, and assayed to determine its uranic acid content m in milligrams. To a sample volume V(ml) was added V, = m/66 + 0.05 V ml of 1 M HCl and 0.1 ml of 5% (v/v) cetylpyridinium chloride. The sample was weighed, centrifuged at 5,000 rpm. (Sorvall SS-34 rotor) for 10 min, and decanted through a plug of glass wool to remove floating particles of precipitate. The supernatant was washed by adsorption on 6.05 M HCl containing 0.01% cetylpyridinium chloride and once in 0.05 M Tris buffer, pH 7, containing 0.01% cetylpyridinium chloride, each resuspension being followed by centrifugation and decanting. The cetylpyridinium chloride was stored for later use or dissolved with warming to 30 to 35° in 2 M NaCl. The collected fraction was termed Fraction v-4-A.
Preparation of Fractions v-4-B and v-4-C - The supernatant from the acid precipitation of Fraction v-4-A by cetylpyridinium chloride was neutralized and buffered by adding 2 V, mmol (see above) of solid Tris. The resulting fine precipitate, which slowly coagulated at room temperature, was centrifuged at 5,000 rpm for 10 min (Sorvall SS-34 rotor), washed twice with 0.05 M Tris buffer, pH 7, containing 0.01% cetylpyridinium chloride, and allowed to drain. The precipitate (Fraction v-4-B) was treated as described above for the washed precipitate of Fraction v-4-A. The supernatant from this precipitation was called Fraction v-4-C.

Preparation of Fractions E-A, E-B, and E-C - Precipitation by cetylpyridinium chloride was also carried out on Sample E dialyzed into 0.02 M NaCl. The procedure followed was essentially similar to that described for precipitation of fractions from Sample v-4. The precipitates from Sample E tended to be more difficult to redissolve in 2 M NaCl than those from Fraction v-4, but dissolution occurred upon addition of GdnHCl to a concentration greater than about 2 M. Fractions E-A, E-B, and E-C refer, as before, to the cetylpyridinium precipitates in acid and neutral media and the supernatant of the latter, respectively.

Proteolytic Digestion - The procedure followed that described by Hascall and Heinogard (3). A weighed amount of crude papain was dissolved in 0.2 M phosphate buffer, pH 6.5, made up to 0.01 M cysteine and activated for 30 min at 37°C. Samples of proteoglycan, previously concentrated by vacuum dialysis in a collodion bag containing in 2 M NaCl, were made 0.01 M in cysteine and incubated with activated papain (0.06 mg/mg of proteoglycan) at 37°C for 2 h. The digest was applied as a volume up to 0.5 ml, containing typically 1 to 2 mg of uronic acid, to a column (9 x 28 cm) of 6% agarose gel (Sepharose 6B) and eluted at 4°C with 0.2 M NaCl at 1.5 ml/h; fractions of about 0.5 ml were collected, weighed, and assayed for uronic acid and protein.

RESULTS AND DISCUSSION

The proteoglycan system extracted from cartilage consists of aggregates of average molecular weight above 2 x 10^7, based on measurements of light scattering (22) or viscosity and sedimentation (23). Hascall and Saidera (21) showed that in 4 M GdnHCl this system is dissociated into proteoglycan "subunits" of average molecular weight 2.5 x 10^6, as well as a "glycoprotein link" aggregating factor. In the following discussion, solvents containing GdnHCl at a concentration of at least 2 M were termed "dissociative" and other solvents "associative." Similar terminology is applied to procedures.

Gel Chromatography of Cartilage Extract - The centrifugation in associative CsCl density gradients was introduced by Franek and Dunstone (20) as a means of separating nonassociated proteins in raw extract from proteoglycan aggregates. This procedure was replaced in our work by gel chromatography in 2 M CaCl2.

FIG. 1. Elution diagram for extract (Sample E) in 2 M CaCl2 of bovine nasal septum cartilage on a preparative 4% agarose (Bio-Gel A-15m) column of about 1650 cm3 bed volume. Eluent: 2 M CaCl2. The ordinate indicates concentration of uronic acid (UA) or protein (UA). In this experiment, 212 mg of uronic acid and 249 mg of protein were eluted from the column. Typically, complete recovery of initial column charge was achieved for these components. Fraction v-4 was constituted by combining the fractions indicated by the brace.

Fig. 1 shows the analytical results obtained from chromatographic fractionation of Sample E on 4% agarose gel with 2 M CaCl2 as eluent. This fractionation clearly separated small nonassociated proteins from a void volume Fraction v-4 (indicated by a brace in Fig. 1), which contained the major part of the proteoglycan. Fraction v-4 comprised 88 ± 3% of the uronic acid and 16 ± 2% of the protein in the original extract and had a protein to uronic acid ratio of 0.27 to 0.32.

In order to compare this material to the product of associative (CsCl) density gradient centrifugation, we fractionated Sample E by the latter method. The densest 10% (V/V) of the gradient, called Fraction A1, comprised 83 ± 5% of the uronic acid and 22 ± 5% of the protein.

Viscometry in 0.2 M NaCl of Fractions v-4 and A1 yielded similar values of [η] ranging from 350 to 400 mls/g in typical preparations. Sedimentation of Fraction v-4 at low ionic strength (normally 0.2 M NaCl) produced schlieren patterns with "fast" and "slow moving" peaks which were qualitatively indistinguishable from those obtained by other workers (10, 21) or by us from Fraction A1. In Fig. 2, values of the sedimentation coefficient s at a number of concentrations for the slow and fast moving peaks of Fractions A1 and v-4 are compared with data from the work of Franek and Dunstone (20). These data indicate that the void volume fraction from chromatography in 2 M CaCl2 is similar in gross composition and in physical properties to Fraction A1 from the CsCl density gradient centrifugation of Sample E. The solid curves are taken from Franek and Dunstone (20), who presented extensive sedimentation data on a sample essentially equivalent to Fraction A1.

Precipitation with Cetylpyridinium Chloride - Precipitation of proteoglycan from Fraction v-4 with slightly less than
Cetylpyridinium chloride precipitation of proteoglycan from acid solutions

Several samples (Samples 1 to 6) of Fraction v-4 and one (Sample 7) of raw extract containing various NaCl and HCl concentrations were precipitated at room temperature by addition of cetylpyridinium chloride (CPC) followed by centrifugation of precipitates. The supernatant solution was neutralized, and a precipitate was obtained which was not present in Fraction v-4. All fractions contained more protein and accompanying hexose than those from Sample v-4. Fractions E-A and E-B did not dissolve completely on warming in 2 M NaCl. This appeared to be due to insoluble hexose- and protein-containing material which was not present in Fraction v-4.

Analytical data for the various crude fractions from Sample v-4 are presented in Table II. These data make clear that by far the major part of the polymeric material precipitates in Fraction A. Hexose and protein occur as significant components of crude preparations of Fraction v-4-B and as major components not precipitated by cetylpyridinium chloride in Fraction v-4-C.

A precipitation with cetylpyridinium chloride in acidic solution directly from Sample E led to similar, although not identical, fractions. Fractions E-B and E-C were slightly larger than those obtained from Fraction v-4; Fraction E-B contained roughly 1% of the uranic acid in Sample E. All fractions contained more protein (and accompanying hexose) than those from Sample v-4. Fractions E-A and E-B did not dissolve completely on warming in 2 M NaCl. This appeared to be due to insoluble hexose- and protein-containing material which was not present in Fraction v-4, evidently being

Table I

Cetylpyridinium chloride precipitation of proteoglycan from acid solutions

<table>
<thead>
<tr>
<th>Sample</th>
<th>Solvent</th>
<th>mg CPC/ mg UA</th>
<th>% UA in supernatant</th>
<th>Average fraction composition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HCl NaCl</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.09 0.12</td>
<td>4.8</td>
<td>0.6</td>
<td>345 253</td>
</tr>
<tr>
<td>2</td>
<td>0.09 0.16</td>
<td>6.6</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.06 0.17</td>
<td>5.7</td>
<td>0.7</td>
<td>390 325</td>
</tr>
<tr>
<td>4</td>
<td>0.07 0.19</td>
<td>5.0</td>
<td>0.7</td>
<td>400 340</td>
</tr>
<tr>
<td>5</td>
<td>0.05 0.04</td>
<td>5.0</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0.05 0.04</td>
<td>5.0</td>
<td>0.8</td>
<td>347</td>
</tr>
<tr>
<td>7</td>
<td>0.04 0.04</td>
<td>5.2</td>
<td>1.1</td>
<td>293</td>
</tr>
</tbody>
</table>

TABLE II

Analytical data for extracts and various fractions from bovine nasal cartilage

For most samples, a range of typical values of percentage composition are presented; otherwise analytical data for a single typical sample are given. The second column gives weight percentage of polymer solute in the sample with respect to the original sample indicated in parentheses. Average compositions refer to weight percentage on the basis of weight of hydrolysis products. Total sample weights were calculated as described under "Analytical Methods."
retarded in the gel chromatographic separation. These insoluble materials usually dissolved readily when the solution was made 2 M or greater in GdnHCl.

Fraction v-4-B could be partially freed of hexose- and protein-containing components by precipitation with methanol, and was freed from both these and contaminating proteoglycan by centrifugation in a dissociative density gradient, as discussed below. Analytical data for a typical product of the methanol precipitation is given in Table II.

Proteolytic Digestion of Fractions—Hascall and Heinegard (3) subjected samples containing proteoglycan aggregates to proteolytic digestion with papain to demonstrate the presence of uranic acid-containing components of high molecular weight by elution in the void volume from 6% agarose gel or, alternatively, in the void volume from Sephadex G-200 after preliminary separation from nonassociated, low molecular weight components on a 2% agarose gel. The component of high molecular weight, which was accompanied by substantial quantities of degraded peptides in void volume fractions eluted in associative solvents, was identified as hyaluronic acid by these authors on the basis of hexosamine composition and behavior on elution from cetylpyridinium chloride cellulose columns.

We examined papain digests of Fractions v-4-A and v-4-B (termed v-4-A-P and v-4-B-P) on 6% agarose gels to characterize them in terms of uranic acid content of the void volume. A calibration on our analytical gel column with Fraction v-4 produced a chromatogram resembling that of Hascall and Heinegard (3). We chose to terminate the void volume fraction at \( V_\text{v} = 0.45 V_\text{v} \), where \( V_\text{v} \) is the bed volume; the volume \( V_\text{v} \) represents the approximate location of the minimum in uranic acid concentration between the initial (hyaluronic acid) peak and the main (chondroitin sulfate) peak in digests of Fraction v-4. Material eluted at volumes less than \( V_\text{v} \) was designated by the suffix -v. There is no detectable uranic acid (i.e., less than about 2 \( \mu g \)/ml) in Fraction v-4-A-P. The data of Hascall and Heinegard (3) show that this result indicates that only chondroitin sulfate (and no hyaluronic acid) is present, so that Fraction v-4-A consists essentially of sulfated proteoglycan.

The elution diagram shown in Fig. 3 for Fraction v-4-BC-P clearly indicates a v-component, which strongly suggests the presence of hyaluronic acid. The persistence of uranic acid into the fractions retarded by 6% agarose is noteworthy. Although a part of this retardation is undoubtedly due to chondroitin 4-sulfate derived from digestion of a small amount of proteoglycan contaminant (see below), a significant hyaluronic acid fraction in 4-B retarded. This result indicates considerable heterogeneity in the cartilage hyaluronic acid, as would be expected from previous fractionations (9, 26, 27) from other sources.

Since chondroitin 4-sulfate appears at significant concentrations at volumes just larger than \( V_\text{v} \), overlap of hyaluronic acid and chondroitin sulfate occurs at such volumes. In addition, Fraction v-4-P-v contained about one-third of its hexosamine as galactosamine, which indicates that some uranic acid from chondroitin sulfate or from incompletely digested proteoglycan contaminated this material. Evidently, use of 6% agarose chromatography as a quantitative assay for hyaluronic acid in digests containing large amounts of chondroitin sulfate must be used with great caution.

Density Gradient Fractionation—To provide further characterization and possibly purification of the B fractions from cetylpyridinium chloride precipitation, we subjected them to density gradient centrifugation under both associative and dissociative conditions. All fractionation data were fitted to an assumed gaussian distribution in the volume to correct the raw data, which give the average concentration over the volume range of a fraction, to the point concentration as a function of volume. Concentration was then plotted against density calculated for the conditions of the experiment with use of the thermodynamic data for CsCl solutions of Iff et al. (28).

Our data for the NaCl-soluble portion of Fraction E-B in an associative CsCl density gradient is shown in Fig. 4. Also shown is a fit of the data obtained in a similar experiment by Silpananta et al. (8) for an unfractonated sample (UFR) of hyaluronic acid from ox synovial fluid.

Data obtained from dissociative density gradients for Fraction v-4-B and for a sample of unfractonated hyaluronic acid from rooster comb (RCH 1) are fitted in Fig. 5. Approximately 10 to 20% of the uranic acid recovered in this experiment was found at densities greater than those allowed by the gaussian fit for hyaluronic acid; this is evidently due to dissociated dense proteoglycan. A substantial component containing most of the protein in the crude fraction separated into the region of density below about 1.38 g/ml.

The fraction which was isolated at densities between 1.30 and 1.51 g/ml, which is appropriate for hyaluronic acid as seen for sample RCH 1 in Fig. 5 and in the data presented for pig skin hyaluronic acid by Mashburn et al. (29), was separated as Fraction v-4-B. In two separate experimental runs 80% and 90% of the uranic acid in Fraction v-4-B appeared in the purified material, which was concentrated by vacuum dialysis in a collodion bag concentrator at 0.2 M NaCl and chromatographed through the G-25 column (see "Preparation of Fraction v-4-A"). Analytical data for this fraction are given in Table III.

The high glucosamine/galactosamine ratio provides chemical evidence for the identification of the glycosaminoglycan component of Fraction v-4-B as essentially hyaluronic acid. The distribution of amino acids in the protein component of the fraction resembles generally, except for the high glycine content, those of the crude protein fractions obtained from an associative CsCl density gradient fractionation (31). The

![Fig. 3. Elution diagram for papain digests on 6% agarose (Sephadex G-11) gel with 0.2 M NaCl as eluent. The left-hand portion (left-hand concentration scale for uronic acid) shows the elution of a digest of Fraction v-4-BC (□) and of a digest of Fraction v-4-A (○), while the right-hand portion completes the diagram of Fraction v-4-A (inset scale compressed to 25× that of left-hand ordinate). Recovery: Fraction v-4-BC, 47 ± 10 μg of uronic acid of 41 ± 2 μg applied; Fraction v-4-A, 126 ± 5 μg of 133 ± 3 μg applied.](http://www.jbc.org/)

3 Details of these calculations are available from R. L. C. upon request.
the ratio of the uranic acid concentration \( c \) at a given density to the maximum concentration \( c^* \) of uranic acid in the gradient. The data were obtained from a sample of the NaCl-soluble portion of Fraction E-B isolated by cetylpyridinium chloride precipitation from dialyzed extract (two tubes: \( \Delta, c^* = 0.066 \text{ mg/ml} \); \( \Delta, c^* = 0.04 \text{ mg/ml} \)) and from the data of Siltapananta et al. (8) for sample UFR (\( M = 14 \times 10^4 \)) of ox synovial hyaluronic acid (\( c^* = 4.5 \text{ mg/ml} \)). The solid lines represent the gaussian distribution which gives a best fit to the data.

---

**Table III**

<table>
<thead>
<tr>
<th>Hexosamines</th>
<th>Weight percentage</th>
<th>Amino acids Residues/1000 residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucosamine</td>
<td>96</td>
<td>Aspartic acid: 80</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>4</td>
<td>Threonine: 35</td>
</tr>
<tr>
<td>Hexuronic acid/hexosamine</td>
<td>1.0 (mole ratio)</td>
<td>Serine: 97</td>
</tr>
<tr>
<td>Hexuronic acid/serum albumin</td>
<td>2.8 (wt. ratio)</td>
<td>Glutamic acid: 122</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Proline: 80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glycine: 125</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alanine: 62</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Valine: 48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methionine: &lt; 5</td>
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<tr>
<td></td>
<td></td>
<td>Isoleucine: 67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leucine: 64</td>
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<tr>
<td></td>
<td></td>
<td>Tyrosine: 12</td>
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<td></td>
<td></td>
<td>Phenylalanine: 30</td>
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<tr>
<td></td>
<td></td>
<td>Lysine: 45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Histidine: 24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Arginine: 44</td>
</tr>
</tbody>
</table>

* Routine ultraviolet analysis gave 15% for this ratio when bovine serum albumin was used as the reference protein.

* Assumed value for purpose of calculating weight of amino acids.

---

**Table IV**

<table>
<thead>
<tr>
<th>Viscosimetric data for v 4 B fractions in 0.2 m NaCl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>v-4-B</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>v-4-B-D</td>
</tr>
</tbody>
</table>

* By ultraviolet analysis.

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Observed in dissociative solvents (Fig. 5) are probably due principally to the fact that the density of hyaluronic acid varies with solvent composition in the GdnHCl mixture and that the mole fraction of GdnHCl decreases significantly at increasing density (29).  

**Physical Properties** — Sedimentation velocity measurements of Fraction v 4 A in 0.2 m NaCl produced single peaked clover leaf patterns with \( s = 20 S \) at 20°, where \( s \) is the value of the sedimentation coefficient \( s \) extrapolated to zero concentration. This result is typical of those found for subunits (21) and indicates that Fraction v-4-A resembles subunits in its sedimentation behavior.

Values of \( (\ln \eta_{rel} / c) \) are given in Table IV for Fractions v-4-B and v-4-B-D. The value of the limiting viscosity number \( [\eta] \) in 0.2 m NaCl, estimated for Fraction v-4-B-D from the tabulated data and Equation 1, is 1072 ml/g. With the approximation that \( [\eta] \) for this fraction may be regarded as due only to the hyaluronic acid component, its viscosity-average molecular weight \( M = M_w \), where \( M_w \) is the weight-average molecular weight, can be estimated from the relation, valid in 0.2 m NaCl (7)  

\[
[\eta] = 0.0228 M^{0.816}
\]

which gives \( M = 5.3 \times 10^6 \). The values of \( (\ln \eta_{rel} / c) \) given in
Table IV for samples of crude Fraction v-4-B, which surely contain more protein, are roughly comparable, when c again is taken to be the hyaluronic acid concentration in the sample. Inclusion of the concentrations of protein (or other contaminants) in c would, of course, lower these estimated values. Sedimentation coefficients at 20° in 0.2 M NaCl are plotted for various samples in Fig. 6. For purposes of comparison expected values of this quantity for hyaluronic acid of M = 5.3 x 10^6 are given as the solid curve, which was calculated as follows. Values of s^0 and the diffusion coefficient D^0 at zero concentration in 0.2 M NaCl were calculated from published equations (7) and were used to construct plots of the diffusion coefficient D (with use of dD/dc = 0.17 x 10^{-4} (7)) and D/s against c, the concentration of hyaluronic acid in grams per milliliter. The values of D/s were calculated from Equation 3 in Ref. 7, for a value of A_2 = 1.8 x 10^{-2} ml g^{-2} and for the values of other quantities in the discussion following Equation 3 in Ref. 7. The solid line of Fig. 6 was obtained from these calculations at each concentration by dividing D/s by D.

The experimental points for the sedimentation coefficient of Fraction v-4-B-D are seen to be in satisfactory agreement with the calculated curve. Other values of s^{-1} plotted for samples known to contain proteoglycan contaminants are smaller, as would be expected from the influence of the faster sedimenting proteoglycan component.

Conclusions —The proteoglycan extracted from bovine nasal septum cartilage is precipitated almost quantitatively from acidic solutions by cetylpyridinium chloride. Neutralization with Tris buffer at ionic strengths less than 0.2 yields a small further precipitate (Fraction B). Comparison of this fraction with samples of known hyaluronic acid by hexosamine analysis, density gradient centrifugation, viscometric, and sedimentation measurements provides a persuasive demonstration that Fraction B is principally hyaluronic acid. Purification in a dissociative density gradient yields a fraction which is estimated to have a molecular weight of about 5 x 10^6, and is polydisperse.

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Isolation and physical characterization of hyaluronic acid prepared from bovine nasal septum by cetylpyridinium chloride precipitation.
R L Cleland and A P Sherblom


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