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 x 10⁶ was estimated for a sample of Fraction B purified in a dissociative (4 M guanidine hydrochloride + 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.

Long chain alkyl cations have frequently been used to precipitate polyanions, particularly glycosaminoglycans (11), by the mechanism once called complex coacervation (2). Recent reports have indicated that the glycosaminoglycan, hyaluronic acid, occurs in cartilaginous tissues, such as bovine nasal septum (3) and pig laryngeal cartilage (4). Extracts of these tissues in solvents of high ionic strength contain uronic acid from hyaluronic acid at levels of 0.5 to 1% of the total uronic acid (3, 5). The major glycosaminoglycan content of these tissues occurs as chondroitin 4-sulfate and keratan sulfate side chains covalently bound to a protein "core" to constitute proteoglycan molecules (6). Selective precipitation of the sulfated proteoglycans with cetylpyridinium chloride in acidic solution was used in this work to isolate hyaluronic acid from cartilage extracts. The molecular weight of the cartilage hyaluronic acid, which had been purified by centrifugation in a dissociative (GdnHCl-CsCl) density gradient, was estimated by viscometry.

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 30 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 1) 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 abbreviation used is: GdnHCl, guanidine hydrochloride.

Designations of particular samples of hyaluronic acid are made to correspond, where possible, to those originally used for these samples: RCH 1, rooster comb hyaluronic acid (7); UFR, ultrafilter residue from ox synovial fluid hyaluronic acid (8). Operational 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 (densest) fraction; A1, 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.
Viscosity — The ratio of flow time of sample to that of solvent was taken as the relative viscosity \( \eta_r \). Flow times were measured at 25°C in a Cannon-Ubbelohde semimicro dilution viscometer. The relative viscosity \( \eta_r \) may be obtained by extrapolation of \( \ln \eta_r/c \) to \( c = 0 \) according to the equation

\[
\ln \eta_r/c = (n - 1)\eta_0/c
\]

where \( k \) lies between 0.10 and 0.15 for most polymer-solvent systems (9). We have consistently found \( k = 0.15 \pm 0.03 \) for hyaluronic acid in 0.2 M NaCl from plots of Equation 1 and have used this value previously to calculate \( \eta_r \) from values of \( \ln \eta_r/c \) at single concentrations for a series of fractions (9). For proteoglycan samples (Fraction A-1-A), we found \( k = 0.00 \pm 0.05 \) to be typical for solutions in 0.2 M NaCl.

Density Gradient Centrifugation — Franck and Dunstone (20) introduced a method for the separation of nonassociated protein from proteoglycan in an associative (CsCl) gradient. This method, and the following centrifugation in a dissociative gradient, composed of a mixture of 4 M GdnHCl and CsCl, used by Hascall and Sajdera (21), were carried out at 45,000 rpm (Beckman 65 rotor) in a Beckman Model L preparative ultracentrifuge maintained at 4°C. Polyallomer tubes were punctured from the bottom, and the contents were removed by suction followed by suspension once with 1.1 ml fraction volumes. Fractions were normally assayed for uronic acid and protein. Densities of fractions were determined by weighing in a calibrated 0.50-ml pipette.

Preparation of Fraction Al — Sample E, made up to a density of 1.71 g/ml by addition of solid CsCl and to a uronic acid concentration of 0.3 mg/ml, was centrifuged for 42 h at 45,000 rpm (Beckman 65 rotor) at 4°C followed by fractionation. A small amount of solid CsCl sedimented as a pellet, but this created no practical difficulty. The densest fraction (AI, density = 1.82 g/ml) was exhaustively dialedyzed into 0.2 M NaCl at 4°C.

Preparation of Fraction v-4 — A 4% agarose gel (Bio-Gel A-15m) was equilibrated with 2 M CaCl₂ and packed into a column (6 x 90 cm). A sample of 50 to 60 ml of Sample E containing about 3 mg/ml of uronic acid was applied to the column and eluted with 2 M CaCl₂ at a flow rate of about 60 ml/hr. Fractions of 50 to 60 ml were collected and precipitated with 2 to 3 volumes of methanol, reprecipitated once in 0.05 M Tris buffer, pH 7, followed by centrifugation and redialyzed into 0.2 M NaCl. The collected fraction was termed Fraction v-4.

Preparation of Fraction v-4A — A 4% agarose gel (Bio-Gel A-15m) was equilibrated with 2 M CaCl₂ and packed into a column (6 x 90 cm). A sample of 50 to 60 ml of Sample E containing about 3 mg/ml of uronic acid was applied to the column and eluted with 2 M CaCl₂ at a flow rate of about 60 ml/hr. Fractions of 50 to 60 ml were collected and precipitated with 2 to 3 volumes of methanol, redissolved in 0.2 M NaCl and reprecipitated. The collected fraction was termed Fraction v-4A.
Preparations 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 Heinegard (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 concentrator, 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 (0.9 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 10^9, based on measurements of light scattering (22) or viscosity and sedimentation (23). Hascall and Sajdera (21) showed that in 2 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 are 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 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 68 ± 5% 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 63 ± 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 ml/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
Hyaluronic Acid of Cartilage

stochastic amounts of cetylpyridinium chloride showed that precipitates contained 0.185 mg of uronic acid/mg of cetylpyridinium chloride (as weighed), which corresponds to 66 g of uronic acid/g equivalent of cetylpyridinium chloride with use of the equivalent formula weight of 358 for the cetylpyridinium (monohydrate) used.

The cetylpyridinium salt of hyaluronic acid is soluble in alkali halide solutions of concentration greater than about 0.2 M at room temperature (1). Antonopoulos et al. (24) eluted hyaluronic acid from cetylpyridinium precipitates on cellulose columns in 0.3 M NaCl at neutral pH. We attempted to isolate hyaluronic acid by selective precipitation of the proteoglycan with cetylpyridinium chloride at 0.3 M NaCl. A solution containing 2.0 mg of Fraction v-4 was made 0.3 M in NaCl with a cetylpyridinium chloride content of 14.7 mg (37% greater than stoichiometric) in a final volume of 2.6 ml. After the voluminous precipitate was centrifuged, no uronic acid could be detected in the supernatant. This is consistent with the finding (10) that cetylpyridinium precipitation at low ionic strength yielded proteoglycan preparations which showed aggregation behavior (fast peaks) in sedimentation. Hyaluronic acid, which is implicated in aggregate formation (3), therefore co precipitates with proteoglycan in 0.3 M NaCl at neutral pH.

However, similar experiments in solutions made 0.05 to 0.1 M in HCl and 0.04 to 0.2 M in NaCl showed that addition of 5.0 mg of cetylpyridinium chloride/mg of uronic acid routinely produced a precipitate whose supernatant was not clouded by further addition of cetylpyridinium chloride. The determination of cetylpyridinium chloride content in the acid supernatant indicated that 0.21 mg of uronic acid precipitated with 1.0 mg of cetylpyridinium chloride under these conditions. The supernatant solution consistently retained 0.5 to 1% of the original uronic acid, as indicated in Table I.

In the following, we wish to show that the uronic acid-containing material in the acidic supernatant solution is essentially hyaluronic acid. Previous experimental work by Scott (25) led us to expect that only nonsulfated glycosaminoglycans would fail to precipitate under acidic conditions.

### Table I

<table>
<thead>
<tr>
<th>Sample</th>
<th>Solvent</th>
<th>mg CPC/mg UA</th>
<th>% UA in supernatant</th>
<th>(γ)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>HCl</td>
<td>NaCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.09</td>
<td>0.12</td>
<td>4.8</td>
<td>0.6</td>
</tr>
<tr>
<td>2</td>
<td>0.09</td>
<td>0.18</td>
<td>6.6</td>
<td>0.7</td>
</tr>
<tr>
<td>3</td>
<td>0.06</td>
<td>0.17</td>
<td>5.7</td>
<td>0.7</td>
</tr>
<tr>
<td>4</td>
<td>0.07</td>
<td>0.19</td>
<td>5.0</td>
<td>0.7</td>
</tr>
<tr>
<td>5</td>
<td>0.06</td>
<td>0.04</td>
<td>5.0</td>
<td>0.8</td>
</tr>
<tr>
<td>6</td>
<td>0.05</td>
<td>0.04</td>
<td>5.0</td>
<td>0.8</td>
</tr>
<tr>
<td>7</td>
<td>0.04</td>
<td>0.04</td>
<td>5.2</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Such species will normally precipitate with cetylpyridinium chloride, however, under neutral conditions at sufficiently low ionic strength (1). The acid supernatant was neutralized by addition of solid Tris (2 mol/mol of HCl used in acidification). When the ionic strength was less than 0.2, a further precipitation occurred to produce a fraction called v-4-B. At higher ionic strength, this fraction did not precipitate on neutralization, but a similar fraction could be precipitated with methanol. The supernatant from either of these precipitations, whose polymeric component was called Fraction v-4-C, was essentially free of uronic acid (see Table II).

Both Fractions v-4-A and v-4-B were soluble in 2 M NaCl or other solvents of comparable or higher ionic strength. The polymeric species were purified by precipitation with 3 or 4 volumes of methanol/volume of solution. After centrifugation, the precipitates were redissolved in 0.2 to 1 M NaCl and reprecipitated with methanol. This procedure removed all detectable cetylpyridinium chloride. To prepare samples for measurements of physical properties, the final precipitate was redissolved in the desired solvent, freed of methanol by dialysis or, preferably, by filtration through a small pore gel, such as Sephadex G-25.

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 uronic 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 II

<table>
<thead>
<tr>
<th>Sample</th>
<th>Original weight percentage</th>
<th>Uronic acid</th>
<th>Hexose</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>percentage on the basis of weight of hydrolysis products</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>mg CPCI</td>
<td>mg UA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Crude</td>
<td>mg</td>
<td>mg</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 (v-4)</td>
<td>98 (v-4)</td>
<td>98 (v-4)</td>
<td>98 (v-4)</td>
</tr>
<tr>
<td></td>
<td>Methanol</td>
<td>0.0 (v-4)</td>
<td>0.0 (v-4)</td>
<td>0.0 (v-4)</td>
</tr>
<tr>
<td></td>
<td>v-4-C</td>
<td>1 (v-4)</td>
<td>&lt;5</td>
<td>50-60</td>
</tr>
<tr>
<td></td>
<td>E-A</td>
<td>96-97 (E)</td>
<td>96-97 (E)</td>
<td>96-97 (E)</td>
</tr>
<tr>
<td></td>
<td>E-B</td>
<td>1-1.5 (E)</td>
<td>18</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>E-C</td>
<td>2-2.5 (E)</td>
<td>2-2.5 (E)</td>
<td>2-2.5 (E)</td>
</tr>
</tbody>
</table>

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 uronic 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 uronic 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 $V_n = 0.45 V_v$, where $V_v$ is the bed volume; the volume $V_n$ represents the approximate location of the minimum in uronic 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 $V_n$ was designated by the suffix -v. There is no detectable uronic acid (i.e., less than about 2 mg/ml) in Fraction v-4-A-P-v. 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 uronic acid into the fraction 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 is also 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 $V_n$, 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 uronic 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 Ifft 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 uronic 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 1.39 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-D. In two separate experimental runs 80% and 90% of the uronic 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-D 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). There

\[ V_n = 0.45 V_v \]

\[ \text{CONCENTRATION (mg/ml)} \]

\[ \text{ELUTION VOLUME (ml)} \]

Fig. 3. Elution diagram for papain digests on 6% agarose (Sephadex G-25) 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 mg of 133 ± 3 mg applied.

[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 $c^*$ is representative of uranic acid in the gradient. The data were obtained from a sample of the NaCl-soluble portion of Fraction v-4-B isolated by cetylpyridinium chloride precipitation from dialyzed extract (two tubes: $\triangle$, $c^* = 0.066$ mg/ml; $\Delta$, $c^* = 0.04$ mg/ml) and from the data of Silpananta et al. (8) for sample UFR ($M = 14 \times 10^4$) of ox synovial hyaluronic acid ($c^* = 4.5$ mg/ml). The solid lines represent the Gaussian distribution which gives a best fit to the data.

![Fig. 4 (left). A fit of experimental data (see text) from an associative (CsCl) density gradient fractionation. The ordinate represents 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 v-4-B isolated by cetylpyridinium chloride precipitation from dialyzed extract (two tubes: $\triangle$, $c^* = 0.066$ mg/ml; $\Delta$, $c^* = 0.04$ mg/ml) and from the data of Silpananta et al. (8) for sample UFR ($M = 14 \times 10^4$) of ox synovial hyaluronic acid ($c^* = 4.5$ mg/ml). The solid lines represent the Gaussian distribution which gives a best fit to the data.](image)

### Table III

Chemical analysis: hyaluronic acid Fraction v-4-B-D

<table>
<thead>
<tr>
<th>Hexosamines</th>
<th>Weight percentage</th>
<th>Amino acids</th>
<th>Residues/1000 residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucosamine</td>
<td>96</td>
<td>Aspartic acid</td>
<td>80</td>
</tr>
<tr>
<td>Galactosamine</td>
<td>4</td>
<td>Threonine</td>
<td>35</td>
</tr>
<tr>
<td>Hexuronic acid/hexosamine</td>
<td>1.0 (mole ratio)</td>
<td>Serine</td>
<td>97</td>
</tr>
<tr>
<td>Hexuronic acid/ amino acids</td>
<td>2.8 (wt. ratio)</td>
<td>Glutamic acid</td>
<td>122</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Proline*</td>
<td>(80)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glycine</td>
<td>159</td>
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<td></td>
<td></td>
<td>Alanine</td>
<td>62</td>
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<tr>
<td></td>
<td></td>
<td>Valine</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methionine</td>
<td>&lt;5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isoleucine</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leucine</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tyrosine</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phenylalanine</td>
<td>30</td>
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<tr>
<td></td>
<td></td>
<td>Lysine</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Histidine</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Arginine</td>
<td>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.

Amino acid analysis indicated a higher protein content than expected. Whether this is contaminating protein incompletely removed by the density gradient separation, or strongly associated protein is not known. We have not attempted further purification. It is noteworthy that ultraviolet absorption based on bovine serum albumin gave a very much lower result for the concentration of aromatic amino acids in Fraction v-4-B-D than in bovine serum albumin.

With respect to the band widths in Figs. 4 and 5, narrower bands are expected for higher molecular weights. Molecular weights of samples indicated in the figure captions show that this expectation is borne out. The somewhat broader bands 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).

### Table IV

Viscometric data for v-4-B in 0.2 M NaCl

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Concentrations</th>
<th>(ln $\eta_{rel}$)/c</th>
</tr>
</thead>
<tbody>
<tr>
<td>v-4-B</td>
<td>0.398</td>
<td>0.27</td>
</tr>
<tr>
<td>v-4-B-D</td>
<td>0.209</td>
<td>0.16</td>
</tr>
</tbody>
</table>

* By ultraviolet analysis.

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 1.072 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 $\bar{M} = \bar{M}_v$, where $\bar{M}_v$ is the weight-average molecular weight, can be estimated from the relation, valid in 0.2 M NaCl (7)

$$[\eta] = \frac{0.0228}{\bar{M}_v^{0.416}}$$

which gives $\bar{M} = 5.3 \times 10^6$. 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 10^5 are given as the solid curve, which was calculated as follows. Values of s^o and the diffusion coefficient D^o 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 mol 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 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 10^5, and is polydisperse.

Acknowledgments - We express our gratitude to William J. Culp and Joan E. Taylor of the Biochemistry Department, Dartmouth Medical School, for their assistance with experiments carried out on their amino acid analyzer.

REFERENCES


Fig. 6. The reciprocal of the sedimentation coefficient s (in S units) in 0.2 M NaCl at 20° as a function of polymer concentration (calculated as the sodium salt of hyaluronic acid). The points refer to data for Fraction v-4-B-D, the purified material between densities 1.39 and 1.51 g/cm^3 from the dissociative density gradient of Fraction v-4 (solid) and the "void volume" Fraction v-4-P-v from a 6% agarose gel (Sepharose 6B) fractionation of a papain digest (v-4-P) of Fraction v-4. The solid line represents the curve of s^-1 expected for the molecular weight 5.3 x 10^5 calculated from published data (7) for hyaluronic acid fractions (see text for details of calculation).
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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