CHONDROMUCOPROTEIN; NEW EXTRACTION METHOD AND ALKALINE DEGRADATION*

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An earlier study showed that from bovine nasal cartilage a product could be isolated consisting of chondroitin sulfate, an equivalent amount of inorganic cation, and protein (1). Three lines of evidence indicated that the polysaccharide and protein were combined as a mucoprotein. It will be convenient to distinguish this as bovine nasal chondromucoprotein.

The extraction method was mild but tedious. Three extractions covering 5 days yielded 20 per cent of the hexosamine of the original cartilage as undegraded mucoprotein. Eight further extractions covering 87 days yielded 22 per cent more of the original hexosamine as partly degraded mucoprotein. Clearly this isolation method could not serve to estimate the total amount of chondromucoprotein in the cartilage and thus the fraction of the total hexosamine of the cartilage that exists in this form.

The present report describes a new method for the extraction of chondromucoprotein from cartilage with a high speed homogenizer which, in a single extraction with water, can yield in 1 day an amount of apparently undegraded chondromucoprotein accounting for over 80 per cent of the total hexosamine initially present. In addition, a study of the alkaline degradation of chondromucoprotein led to a greatly simplified method for the preparation of chondroitin sulfate as a crystalline barium salt.

Methods and Results

In a room at 4° fresh bovine nasal cartilage was freed of all perichondrium, rinsed with water, and diced with a razor blade to give pieces about 2 to 5 mm. on a side. The fresh cartilage was generally used within 24 hours after its preparation, but it could be kept at 4° with the addition of a small amount of thymol for as long as 4 days with no observable effects on the products isolated. From this diced fresh cartilage dried cartilage was prepared in two ways. The first, made by drying the diced fresh cartilage

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CHONDROMUCOPROTEIN

with acetone and removing all solvent in vacuo, is called diced dried cartilage. The second was made by putting the fresh wet cartilage (10.0 gm.) in the baffled flask (500 ml.) of the VirTis 45 homogenizer with ethanol (300 ml.) and running at top speed for 10 minutes. The disintegrated cartilage and alcohol were centrifuged and the residue was washed with ethanol and ethyl ether and dried in vacuo. This material is called powdered dried cartilage. It is a fine, white, fluffy powder more suitable for analytical procedures. By either method the dry weight of the cartilage lay in the range 23 to 25 per cent of the wet weight on several dozen preparations. Analysis of several samples of the powdered dry cartilage gave values, as per cent, within the ranges; N (Kjeldahl), 8.3 ± 0.5; hexosamine (2, 3), 9.4 ± 0.3; S (total), 2.4 ± 0.1; hydroxyproline (4), 5.1 ± 0.4; proline (5), 5.4 ± 0.6; moisture; 9.5 ± 0.5; ash (as sulfate), 9.2 ± 0.9.

For the extraction of chondromucoprotein the cartilage was disintegrated in the homogenizer by stirring with water at top speed (rated at 45,000 r.p.m.). The two main factors that were found to control the amount extracted were the proportion of water to cartilage and the time homogenization was carried on. It seemed to make no difference whether fresh or dried cartilage was used, provided the dried cartilage was rehydrated before beginning the extraction. To rehydrate the dried cartilage it was allowed to stand with water (40 ml. per gm. of cartilage) for 16 hours for the diced material and 1 hour for the powdered material. Use of the dried cartilage instead of the fresh cartilage is of advantage when a series of comparative studies is undertaken, since the dried cartilage can be stored indefinitely.

For the preparation of the chondromucoprotein two procedures will be described differing only in details and having different objectives. Method A is useful when it is desired to prepare chondromucoprotein in large amounts with no concern about extracting the maximal yield from a given amount of cartilage. In Method B what appears to be all the extractable chondromucoprotein can be extracted, but the procedure is more time-consuming and allows only small amounts of cartilage to be worked up at a time.

Method A—Diced fresh cartilage (10.0 gm.) or rehydrated dried cartilage (2.5 gm.) and water (300 ml.) were put in the 500 ml. baffled flask of the VirTis 45 homogenizer equipped with two spaced blades and the flask was set in an ice bath. The homogenizer was run at top speed for 15 minutes while constantly replenishing the ice of the bath. This yielded a viscous opalescent fluid often containing pebbles of cartilage not disintegrated. The temperature of the mixture was 10–15°C. To this, ethanol (600 ml.) was added and the mixture was centrifuged at 2000 r.p.m. for 40 minutes. The supernatant liquid was generally clear, though occasionally small amounts of flocculent material persistently floated. The liquid was filtered through a plug of glass wool. The residue, a soft mass of flocculent
material, was washed by stirring with an equal volume (270 ml.) of ethanol and centrifuging again. The clear supernatant liquid was filtered through the glass wool plug and added to the main volume of clear liquid. The residue, washed on the centrifuge several times with ethanol and ethyl ether and dried in vacuo, weighed 1.60 to 1.86 gm. It had a hexosamine content of 5.2 to 6.2 per cent.

The main volume of clear liquid which had been filtered through glass wool had a volume of about 1 liter and was very faintly opalescent. The chondromucoprotein could be precipitated from this solution by addition of salts. For this purpose potassium acetate was preferred because it is alcohol-soluble, and 10 gm. were added, producing an immediate flocculent precipitate of the potassium salt of the chondromucoprotein. This was centrifuged, washed several times with ethanol, then with absolute ether, and dried in vacuo over CaCl₂. The perfectly white product weighed 0.55 to 0.80 gm. Because of the short homogenizing time and the relatively small volumes to be centrifuged, as many as a dozen such batches can be completed in a day, giving a total yield of about 4 gm.

Method B—Rehydrated dried cartilage (amounts between 0.5 and 2 gm.) was homogenized in the VirTis 45 with water (280 ml.) for 30 minutes at top speed, with cooling as before. Stirring was continued for another 30 minutes at the slowest speed of the VirTis 45 and then for a final 30 minutes at the top speed. The temperature of the opalescent liquid should not be over 18°. Ethanol (700 ml.) was then added. No visible pebbles of cartilage remained. The further isolation procedure was exactly as described for Method A. Method B differs from Method A in that a longer time for homogenization is used.

Yields of both the chondromucoprotein and the residue of cartilage matrix left after the extraction are summarized in Table I. The yields of chondromucoprotein by Method A given in Lines 2 and 3 of Table I were greater than those obtained in partly degraded form after 2.5 months of extraction by the method previously described (1) and indicated in Line 1 of Table I. Even higher yields of the chondromucoprotein can be obtained by increasing the length of time of homogenization (Method B) and the proportion of water used as shown in Lines 4 to 7 of Table I. A practical limit to the amount of chondromucoprotein extractable seems to be reached under the conditions shown in Line 6, since the product obtained under the conditions of Line 7, although greater in amount, is no longer completely soluble in water and contains small amounts of hydroxyproline (<0.9 per cent). Thus Line 6 represents the conditions for obtaining the highest yield of mucoprotein having the composition, solubility, and viscosity previously described. This highest yield is nearly half the dry weight of the cartilage.

The chondromucoprotein made by either Method A or B, with the
exception of the preparations represented by Line 7 of Table I, on analysis gave the following values as per cent: hexosamine, 17.4 ± 0.4; nitrogen, 5.25 ± 0.15; sulfur, 3.9 ± 0.3; hydroxyproline, 0; proline, 1.0 ± 0.1; moisture, 8.0 ± 0.1; ash as sulfate, 20.6 ± 0.8. Agreement with earlier values (1) is close. This product can be purified by reprecipitation as described in the earlier work. This has no observable effect on the analytical values.

The composition of the insoluble residue left after the extraction of chondromucoprotein from cartilage depends of course on the amount extracted. The residue left after extraction by Method B, Line 6, gave the following analytical values as per cent: hexosamine, 3.5 ± 0.5; nitrogen, 12.3 ± 1.3; sulfur, 1.2 ± 0.2; hydroxyproline, 10.0 ± 0.3; proline, 10.5 ± 0.4; moisture, 8.8 ± 1.2; ash as sulfate, 2.2 ± 0.1.

Extraction by Method B, as represented by Line 6, thus separates cartilage almost quantitatively into two parts of nearly equal weight, a water-soluble mucoprotein and a water-insoluble residue. Table II summarizes the distribution of some of the components of cartilage between these two parts. Judging by its hydroxyproline content, the residue contains nearly 75 per cent collagen. The remaining components of the residue may be in part unextracted chondromucoprotein and in part a neutral polysaccharide for which evidence has been cited by Glegg, Eidinger, and Leblond (6). It is not clear why attempts to press the extraction of the chondromucoprotein further, as represented by Line 7, Table I, yield a product not completely soluble in water and containing hydroxyproline.

Because the yield of chondromucoprotein is so high, approaching the total that could exist in the cartilage, some comparisons were made of the

### Table I

<table>
<thead>
<tr>
<th>Line No.</th>
<th>Cartilage at start</th>
<th>H₂O used, ml. per gm. dry cartilage</th>
<th>Method</th>
<th>Yield CMP, gm. per gm. dry cartilage</th>
<th>Yield RES, gm. per gm. dry cartilage</th>
<th>Total recovery, per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wet</td>
<td></td>
<td>(1)*</td>
<td>0.200</td>
<td>0.702</td>
<td>97.4</td>
</tr>
<tr>
<td>2</td>
<td>Dry</td>
<td>120</td>
<td>A</td>
<td>0.272</td>
<td>0.706</td>
<td>94.2</td>
</tr>
<tr>
<td>3</td>
<td>Wet</td>
<td>120</td>
<td>A</td>
<td>0.236</td>
<td>0.706</td>
<td>94.2</td>
</tr>
<tr>
<td>4</td>
<td>Dry</td>
<td>135</td>
<td>B</td>
<td>0.348</td>
<td>0.557</td>
<td>90.5</td>
</tr>
<tr>
<td>5</td>
<td>&quot;</td>
<td>180</td>
<td>B</td>
<td>0.401</td>
<td>0.551</td>
<td>95.2</td>
</tr>
<tr>
<td>6</td>
<td>&quot;</td>
<td>280</td>
<td>B</td>
<td>0.462</td>
<td>0.495</td>
<td>95.7</td>
</tr>
<tr>
<td>7</td>
<td>&quot;</td>
<td>540</td>
<td>B</td>
<td>0.522</td>
<td>0.387</td>
<td>90.9</td>
</tr>
</tbody>
</table>

* Bibliographic reference.
properties of the material prepared by the new procedure with that prepared by the mechanically milder earlier method. It was pointed out previously (1) that, if a solution of chondromucoprotein in water was filtered on a sintered glass bacterial filter (pore diameter about 1 μ), none of the mucoprotein passed through the filter and that this seemed to be a measure of the absence of degradation of the chondromucoprotein. The potassium salt of chondromucoprotein prepared by the new procedure when dissolved in water is also completely non-filtrable. It has further been found non-filtrable when dissolved in 1 M KCl or 5 M CaCl₂. This is evidence that protein and polysaccharide are bound together and that this binding is not salt-like. Dissolved in 10 M urea the chondromucoprotein becomes partly filtrable. To learn the amount and composition of the filtrable part the following experiment was set up. Chondromucoprotein (0.34 gm.) dissolved in 10 M urea (50 ml.) was filtered with suction on a Morton type, Pyrex glass bacterial filter at 37° for a week. The syrupy residue on the filter was stirred with 10 M urea (35 ml.) and filtration was continued for another week. The residue on the filter, dissolved in water (50 ml.), was precipitated with ethanol (150 ml.) and potassium acetate (1 gm.). The flocculent precipitate, washed and dried, weighed 0.30 gm. and had 4.7 per cent N and 16.4 per cent hexosamine. To the solution that had passed through the bacterial filter, potassium acetate (10 gm.) and ethanol (1250 ml.) were added. The flocculent precipitate, washed and dried, weighed 0.04 gm. and had 9.4 per cent N and 12.4 per cent hexosamine. Thus in the presence of 10 M urea about 10 per cent of the mucoprotein becomes filtrable and this fraction is richer in protein than chondromucoprotein.

**Table II**

<table>
<thead>
<tr>
<th>Amount in 1 gm. of dry cartilage</th>
<th>Amount recovered in CMP</th>
<th>Amount recovered in RES</th>
<th>Total recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>1000 mg.</td>
<td>460 mg.</td>
<td>490 mg.</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>95 mg.</td>
<td>80 mg.</td>
<td>17.0 mg.</td>
</tr>
<tr>
<td>Sulfur</td>
<td>25 mg.</td>
<td>0 mg.</td>
<td>0 mg.</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>51 mg.</td>
<td>72 mg.</td>
<td>4.9 mg.</td>
</tr>
<tr>
<td>Proline</td>
<td>55 mg.</td>
<td>0 mg.</td>
<td>0 mg.</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>83 mg.</td>
<td>20 mg.</td>
<td>60 mg.</td>
</tr>
</tbody>
</table>

*Distribution of Yield and of Several Components of Dry Cartilage between Water-Soluble Chondromucoprotein (CMP) and Water-Insoluble Residue (RES) When Separation Is Made by Method B (Line 6, Table I)*
The ultraviolet absorption spectra of the chondromucoprotein in water, acid, and alkali are shown in Fig. 1. The high absorption at 280 μm had been used in earlier work (3) to follow the purification of chondroitin sulfate at a time before the existence of the mucoprotein was suspected. The curve for the alkaline solution (Curve B) can be used to calculate the tyrosine and tryptophan contents by the method summarized by Beaven and Holiday (7). The results of such calculations are 1.0 per cent for tyrosine and 0.6 per cent for tryptophan. In the earlier study (1) a tyrosine content of 6 per cent was estimated based on the use of the Folin-Ciocalteu reagent. The Folin-Ciocalteu value is probably rendered high by the large amount of reducing sugar in the hydrolysate. That the earlier value was too high has already been pointed out by Mathews and Lozai-tyte (8). The higher absorption shown by the curve for the acid solution (Curve A) is mainly due to production of a marked opalescence as is apparent from the high optical density at 340 to 360 μm. No visible precipitate can be seen, however, in this acid solution.

Because of the large amount of chondromucoprotein present in cartilage and the great effect the viscosity of the ground substance may have on the physical properties of cartilage, a study was made of the variation in viscosity of chondromucoprotein with its concentration in buffered salt solution. The buffered salt solution used was that recommended by Hadidian and Pirie (9), 0.05 M NaCl and 0.05 M phosphate at pH 7.0. Preparation of a stock solution of the potassium salt of chondromucoprotein at a concentration of 1 per cent in this buffer required an hour of constant stirring. The viscous solution was opalescent and was centrifuged to remove air bubbles. Dilutions were made in the same buffered salt solution and viscosities were measured in Ostwald viscosimeters containing 2 to 3 ml. of liquid and flow times for water of 20 to 30 seconds at 24.3°. Freshly made solutions of chondromucoprotein surprisingly showed a progressive drop in viscosity with time. With solutions whose concentrations were below 0.5 per cent, this was scarcely noticeable in a few hours, but at higher concentrations the phenomenon became very striking. Because of the long time required to dissolve the mucoprotein, the first viscosity measurement could not be made earlier than 2 hours after beginning to dissolve it. In Fig. 2 are presented the results; Curve A shows the relative viscosity of solutions 2 hours after the addition of buffer to the dry mucoprotein; Curve B shows the relative viscosity 20 hours later. To find out if any permanent and irreversible alteration in the chondromucoprotein was involved, solutions were allowed to stand at room temperature for 1 or 2 days, the mucoprotein was then precipitated by addition of potassium acetate and alcohol, and the flocculent precipitate was washed with alcohol and ether and dried in vacuo. On dissolving the
product in the buffered salt solution, the same sequence of changes in 
viscosity was repeated starting at the same initial value of the relative 
viscosity. The progressive drop in viscosity probably reflects some change 
that continues after the mucoprotein has gone into solution, perhaps a 
change in its state of hydration or in the shape of the molecules in solution.

![Figure 1: Absorption spectra of the potassium salt of chondromucoprotein (0.30 
per cent solution). Curve A, 0.1 M HCl; Curve B, 0.1 M NaOH; Curve C, water. 
Curve D for comparison is a 0.30 per cent solution of purified potassium chondroitin 
sulfate in water. Ordinate, optical density observed in a 1 cm. cell; abscissa, wave 
length in millimicrons. The solution for Curve A was strongly opalescent; thus the 
high optical densities observed for it are largely due to light scattering (see the text).

Fig. 2. Relative viscosity (ordinate) as a function of the concentration of the 
potassium salt of chondromucoprotein (abscissa) in phosphate buffer (0.05 M) con-
taining NaCl (0.05 m) at pH 7.0. Curve A, solution 2 hours old; Curve B, solution 
20 hours old; Curve C, solution heated at 65° for 5 minutes. All viscosities are meas-
ured at 24.3°.

At higher temperatures the change progresses more rapidly and at 65° ap-
ppears to be complete in 5 minutes, since, after such heating, measurements 
of viscosities at 24° were found to be constant with time. Curve C of 
Fig. 2 shows relative viscosities at 24.3° of chondromucoprotein solutions 
that have been heated for 5 minutes at 65° in the buffered salt solution.

Chondroitin sulfate has generally been prepared from cartilage by al-
alike extraction followed by a complex set of procedures designed to 
remove contaminating protein. These procedures have included the use of
protein precipitants, adsorption of protein to a variety of earths, gel forma-
tion of protein with chloroform and amyl alcohol, and precipitation of the
polysaccharide from water with a large volume of glacial acetic acid. 
Since most of the chondroitin sulfate of cartilage can now be separated in
the form of chondromucoprotein by a method that is chemically mild, it
seemed worthwhile to try to prepare the chondroitin sulfate from the
mucoprotein after a treatment with alkali. Of the many procedures
tried, the following was most satisfactory.

The potassium salt of chondromucoprotein (2.0 gm.) stirred with sodium
hydroxide solution (150 ml., 0.18 M) gave a clear colorless solution which
was allowed to stand at 37° for 20 hours. The solution remained clear but
turned faintly yellow. Glacial acetic acid was added (1.7 ml.), bringing
the pH to 5.70, and the solution was dialyzed to remove the inorganic
sulfate liberated. Barium chloride (2 gm.) was added, the solution volume
was measured, and exactly one-quarter of this volume of ethanol was
added. A small amount of flocculent precipitate formed which was
centrifuged and discarded. Addition of ethanol to the clear liquid was
continued, bringing the ethanol concentration to 50 per cent and producing
a dense white precipitate of crude barium chondroitin sulfate. This was
centrifuged and dissolved in water (150 ml.), barium chloride (1 gm.)
was added, and ethanol (38 ml.) was stirred in to bring the concentration
to 20 per cent. A small amount of flocculent precipitate was again pro-
duced which was removed by centrifugation and discarded. To the clear
solution more ethanol was added (62 ml.), bringing its concentration to
40 per cent and again producing a dense white precipitate which was
centrifuged. The pure white product was dissolved in water (150 ml.)
and barium chloride (1 gm.) was added. Addition of ethanol to 20 per
cent now produced no precipitate, but precipitation began sharply with the
least further addition of ethanol. Addition of ethanol was continued at
intervals over several hours until a total of 75 ml. had been added. If
the addition was sufficiently slow, the product was sometimes crystalline
at this point. It was centrifuged, washed with alcohol and ether, and
dried in vacuo. The yield was 1.16 gm. and the product was perfectly
white. More perfectly formed crystals can be produced by repeating the
precipitation process as described above. Under the microscope at high
power the crystals appear as aggregates of plates. The following analytical
data (in per cent) were found: C, 24.43; H, 4.77; N, 1.99; S, 4.62; ash as
sulfate, 33.8; hexosamine, 20.2; moisture, 10.0. The values calculated
(in per cent) for \(\text{C}_{14}\text{H}_{19}\text{O}_{14}\text{NSBa} \cdot 5\text{H}_{2}\text{O}\) are C, 24.7; H, 4.25; N, 2.05; S,
4.69; ash as sulfate, 34.1; hexosamine, 26.2; moisture, 13.2. For a solution
(2.5 per cent) in water, \([\alpha]_{D}^{25}\) was found to be \(-20.1°\). The amount of
inorganic sulfate liberated during the course of the alkaline treatment
described above was measured in a separate experiment by adding barium
chloride after acidification with acetic acid and weighing the barium sulfate precipitated. This amounted to 40 mg. (170 μmoles). The yield of barium chondroitin sulfate in such an experiment was 1.10 gm. (1600 μ periods); hence the amount of sulfate liberated is about 10 per cent of the sulfate in the chondroitin sulfate isolated.

As in previous work on the preparation of chondroitin sulfate (3, 10), the hexosamine found was far below the calculated value. On the other hand, the values found for C, H, N, S, and ash are close to the calculated values for these elements. In view of the close agreement of values for elementary analysis with those calculated it seems that the low value found for hexosamine must be attributed to a fault in the analytical method. An obvious source of error is the use of glucosamine hydrochloride as the standard for colorimetric estimation of the hexosamine of cartilage, chondromucoprotein, and chondroitin sulfate in which the hexosamine is thought to be galactosamine though a recent report (11) suggested the presence of talosamine also. The ratio of hexosamine found (20.2) to hexosamine calculated (26.2) for barium chondroitin sulfate is 0.77. The value for this ratio found by analysis of the hexosamine hydrochloride prepared from chondroitin sulfate was 0.80. Thus, if the hexosamine isolated from chondroitin sulfate were used as the standard, then the expected value would be found for the per cent of hexosamine in the barium chondroitin sulfate.

DISCUSSION

The new method for the isolation of chondromucoprotein from cartilage raises some new thoughts. A complete mechanical disintegration of the cartilage structure seems to be the most important requirement for extraction of large amounts of the mucoprotein. Even with a high speed homogenizer, a rather long time (up to 1 hour) and a large amount of water (up to 300 ml. per gm. of dry cartilage) must be used to approach complete extraction (50 per cent of the dry weight of the cartilage). By contrast, in the absence of such disintegration Jorpes (12) found that only traces of sulfur-containing substances were extracted from beef tracheal cartilage by water. Hass and Garthwaite (13) in carefully done experiments found that, when thin sections (30 μ) of infant epiphyseal cartilage were treated with 1 per cent NaCl or buffers at pH 6 or 8, only 6 per cent of the dry weight of the cartilage was extracted. They concluded that the chondroitin sulfate of cartilage could be completely extracted only at or above pH 12. Similar conclusions have been reached for machine-ground bovine nasal cartilage (3). In view of the solubility in water of chondromucoprotein and the absence of any visible membrane barriers in the cartilage matrix it seems odd that so little mucoprotein can be extracted by water without disintegration of the cartilage and violent mechanical agitation in
large volumes of water. An observation that may be relevant has been made by Ludwig¹ and checked in this laboratory. Dissolved in buffer at pH 7 and subjected to electrophoresis on paper, chondromucoprotein does not move at all while chondroitin sulfate under these conditions moves rapidly. Yet on electrophoresis in free solution both chondromucoprotein and chondroitin sulfate move with almost the same speed. It seems possible that the fibers of the paper act mechanically to entangle the high molecular weight mucoprotein and prevent its movement but do not hinder motion of the much smaller chondroitin sulfate. The collagen fibers of cartilage may act similarly to prevent diffusion of the chondromucoprotein into solution unless the collagen network is disintegrated and the shreds violently agitated in a large volume of water. This thought may have bearing on the structure of cartilage.

The viscosities of solutions of the chondromucoprotein (Fig. 2) become particularly impressive, considering the possible concentration of the protein in the ground substance of cartilage. 100 gm. of fresh wet cartilage contain about 75 gm. of water and 12.5 gm. of chondromucoprotein; thus the concentration of the latter might be about 16 per cent.

SUMMARY

A method is described by which the chondromucoprotein of fresh or dried beef nasal cartilage can be extracted with water in yields up to 50 per cent of the dry weight of the cartilage. This yield accounts for 80 per cent of the total hexosamine of the original cartilage. The essential feature of the method is the thorough disintegration of the cartilage in a large volume of water by the use of a high speed homogenizer. A simple method is described for degrading this chondromucoprotein to give chondroitin sulfate as the crystalline barium salt in a yield of over 75 per cent of the amount contained in the chondromucoprotein.

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