ISOLATION OF HYALURONATEPROTEIN FROM HUMAN
SYNOVIAL FLUID *

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The high molecular weight components of synovial fluid are hyaluronate and proteins. Hyaluronate is synthesized by cells of the synovial membrane, and its concentration in normal human synovial fluid is about 3 mg per g (1). Proteins in synovial fluid appear to be identical with those in serum (2). The protein concentration in normal synovial fluid is about 20 mg per g. A problem of considerable interest is whether hyaluronate in serum (2) and synovial fluid is free or exists as a compound with protein. To answer this question, electrophoresis or ultrafiltration have been used in attempts to separate hyaluronate from the proteins of synovial fluid. Some samples of hyaluronate, isolated from human or bovine synovial fluid by electrodialysis at pH 7.2, and treated with Celite to remove protein impurities, had a molar ratio of nitrogen to hexosamine of 1.00 and so were apparently protein-free (3, 4). Hyaluronate's enormous molecular weight (1 × 10^6 or more) and even greater molecular volume compared to proteins of synovial fluid account for the retention of hyaluronate on special filters that allow proteins to pass through. In contrast to the products obtained by electrodialysis, hyaluronate isolated from bovine synovial fluid by 19 consecutive ultrafiltrations on sintered glass contained 25 per cent protein (5). It was thought that this protein was firmly bound to hyaluronate, and necessary for the anomalous viscosity of hyaluronate solutions. Later it was shown that over 65 per cent of the protein in this product could be removed by trypsin digestion without altering hyaluronate's anomalous viscosity (6).

The state of the hyaluronate in native synovial fluid needs to be clarified. Some properties of the hyaluronate might depend on the presence of a firmly bound protein moiety, as, for example, the physical properties of chondromucoprotein isolated from bovine nasal cartilage are due to the combination of chondroitin sulfate and a noncollagenous protein. This paper describes the isolation of hyaluronate from normal human synovial fluid by either ultrafiltration or zone electrophoresis. The final products were treated with adsorbents to remove protein impurities. Isolated by either method, hyaluronate contained about 2 per cent protein. This quantity of protein could not be detected regularly by molar ratios of nitrogen to glucosamine, and special methods were required to confirm its presence. This protein was also shown to be firmly combined with the hyaluronate. Objections have been raised to the use of terms such as "mucoprotein" or polysaccharide-protein "complex," and it has been suggested that "proteimpolysaccharide" be used (7). In this paper the compound isolated from synovial fluid will be termed "hyaluronateprotein" (HP).

**EXPERIMENTAL METHODS**

1. Synovial fluid was obtained with sterile techniques from subjects who died of acute injuries and who were apparently free of joint disease. Some fluids were obtained from living subjects with normal joints. Fluids were stored at 5°C and used within 1 week. The protein content of these fluids was less than 25 mg per g.

2. Isolation of HP by ultrafiltration. A combination of ultrafiltration, adsorption, and ultracentrifugation was used to isolate HP from pooled synovial fluids. Progressive removal of proteins was most simply followed by determining the ratio mg protein/mg hyaluronate (hexuronic acid × 2.5 = hyaluronate). Adsorption was carried out by passing synovial fluid through a column of IRC-50 and hydroxylapatite. Ultrafiltration of fluids

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on sintered glass was a prolonged process, and much more rapid filtration was achieved by using special cellulose filters and glass filtration flasks.\(^1\) Hyaluronate was found to pass through filters ranging in pore diameter from 5 \(\mu\) to 0.22 \(\mu\), but was retained completely on 0.1 \(\mu\) filters. Synovial fluid proteins filtered slowly through the viscous gel of hyaluronate retained on the 0.1 \(\mu\) filter, and were not removed completely by a single ultrafiltration. Accordingly, the hyaluronate was dissolved and ultrafiltered at least 4 times. Ultracentrifugation of the ultrafilter residue was carried out to remove traces of insoluble protein. In our experience, the most suitable procedure for the isolation of hyaluronate from large quantities of human synovial fluid is as follows.

Samples of synovial fluid were pooled to provide about 20 g, and this was diluted 1:10 with a pH 7.2 phosphate-NaCl buffer (0.04 M phosphate, 0.10 M NaCl). The diluted fluid was dialyzed for 24 hours at 5° C against 2500-ml changes of the buffer. The column for adsorption was prepared as follows: 50-ml equal volumes of packed hydroxyapatite (8) and IRC-50 resin were equilibrated with the buffer, mixed, and added to a glass tube 2.5 \(\times\) 50 cm stopped with a plug of cotton wool. The mixture settled without pressure, forming a column 2.5 \(\times\) 16 cm that was kept at 5° C. The dialyzed, diluted synovial fluid was added to the column and eluted with the buffer. The rate of flow through the column was kept constant by maintaining the level of buffer 20 cm above the column of adsorbents. The large volume of eluate from the column, about 500 ml, was divided among 6 filtration flasks containing Millipore filters of 0.1 \(\mu\) pore diameter. These flasks were evacuated to about 1 mm Hg and kept at 5° C. When ultrafiltration was complete, at about 36 to 48 hours, the material retained on the filter was washed by adding 10 ml of the buffer and the flasks were re-evacuated. When this added buffer was filtered completely, the filter was placed in a 100-ml glass beaker containing about 15 ml of phosphate-NaCl buffer. The viscous mass of hyaluronate was scraped off the filter with a plastic rod, and the beaker was shaken at 5° C for 24 hours to dissolve the hyaluronate. The viscous fluid was then ultracentrifuged (Spinco) 30 minutes at 105,000 \(\times\) G to remove insoluble material. The sediment was washed with buffer and ultracentrifuged again. The supernatant fluids were combined and ultrafiltered. The process of ultrafiltration and ultracentrifugation was carried out 3 or 4 times. Part of these final products was dialyzed against KAc (0.15 M) and the HP precipitated with 3 volumes of ethanol at 5° C, washed with ether, and dried over P\(_2\)O\(_5\) in a vacuum.

3. Isolation of HP by zone electrophoresis. HP was isolated from small quantities (1 g) of normal human synovial fluid by zone electrophoresis at pH 5.4 by methods previously described (9). Protein impurities were removed by adsorption with IRC-50 resin and hydroxyapatite.

4. a) Labeling with \(^{131}\)I. To a test tube 13 \(\times\) 100 mm, the following were added in order: 0.05 ml KI (0.01 M), 10 to 100 \(\mu\)c \(^{131}\)I, 0.2 ml HCl (2.5 N), 0.05 ml Na\(_2\)NO\(_3\) (1 M), and 0.5 ml NaOH (1 N). The contents were mixed, adjusted to pH 8.0, and added rapidly with constant stirring to a solution of HP, usually 1 to 2 g with 1.25 mg HP per g, which had been previously dialyzed 8 hours against 2 250-ml changes in a pH 8.1 borate buffer (1 part 0.16 M NaOH added to 5 parts 0.16 M NaCl and 0.2 M boric acid). The mixture was incubated at room temperature for 10 minutes. The free iodine was removed by dialysis at 5° C for 48 hours against 4 1000-ml changes of a pH 7.2 buffer (.15 M NaCl, 0.01 M phosphate).

b) Labeling with \(^{51}Cr\). \(^{51}Cr\), 50 \(\mu\)c, was added to hyaluronate that had been dialyzed in the .04 M phosphate-NaCl buffer. The solution was mixed and incubated at room temperature for 1 hour. Free \(^{51}Cr\) was removed by dialysis at 5° C in the buffer.

5. Zone electrophoresis of labeled products. Polyvinyl chloride (Geon resin 427) \(^2\) was washed for 2 days with NaCl (0.15 M) and for 1 day in the buffer used for electrophoresis. The slurry was poured into a 21 \(\times\) 13 \(\times\) 0.5 cm plastic mold that was subdivided longitudinally into five compartments, each 21 \(\times\) 2 \(\times\) 0.5 cm, by glass rods wrapped in rubber tubing. Double-thickness Whatman no. 1 filter paper was used as wicks. The 0.5 ml samples were added to a small .02 \(\times\) 1 \(\times\) .05-cm slit 4 cm from the cathodal end of each compartment. Five samples could be run simultaneously. Electrophoresis was performed at a voltage gradient of 15.5 volts per cm at 5° C for about 6 hours. After electrophoresis, the block was blotted dry, each compartment was cut into 1-cm segments, and each segment was placed in a test tube 13 \(\times\) 100 mm. The \(^{131}\)I in each segment was determined by counting in a deep well scintillation counter. To each tube, 1 ml of the .04 M phosphate-NaCl buffer was added. The contents were mixed with a glass rod, and after centrifugation at 2500 rpm for 10 minutes, the supernatant fluid was analyzed for hexuronic acid.

6. Chromatography. Ascending methods were carried out in stainless steel chambers \(^3\) 23 cm long, 9 cm wide, and 23 cm high with Whatman no. 1 paper 20 cm square.

Amino acids. HP was hydrolyzed in 6 N HCl in a sealed tube for 17 hours at 105° C and the hydrolysate evaporated to dryness in a vacuum over NaOH. The residue was dissolved in water, filtered through glass wool, evaporated to dryness, and dissolved in distilled water. Two ascents were made in butanol-acetic acid-water (12:3:5) (11) and a single ascent in a second dimension with a phenol solvent (12). Amino acids were detected with 0.25 per cent weight per volume of 1,2,3-indantriione hydrate (Ninhydrin) in acetone.

Neutral sugars and nucleotides. Hydrolysis was carried out in 0.5 N HCl in sealed tubes for 16 hours at 94° C and the hydrolysate evaporated to dryness. One or two ascents were made in isopropanol-water (80:20) (11). Nucleotides were detected by ultraviolet light

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\(^1\) Millipore Corp., Bedford, Mass.

\(^2\) B. F. Goodrich Chemical Co.

\(^3\) A. H. Thomas, Phila., Pa.
with a 2570 Å lamp. Amino and neutral sugars were located by an aniline reagent (12), and N-acetylamino sugars by the method of Salton (13). The isopropanol solvent proved very useful; it did not diminish ultraviolet absorption of nucleotides as did solvents containing butanol, and it separated galactosamine and glucosamine.

7. Viscosity. All samples were first dialyzed in the .04 M phosphate-NaCl buffer. Samples were filtered through 5 µ Millipore filters to remove threads or particles. To measure relative viscosity, an Ostwald viscometer (2-ml capacity) was used with a flow time for the buffer of 180 seconds in a constant temperature bath at 25° C ± 0.1° C. Anomalous viscosity was determined by a modification of the method of Levine and Kling (14) and the results were plotted according to methods of Barnett (15).

8. Ultracentrifugation. Analysis of HP (1.0 mg per g in .04 M phosphate-NaCl buffer, pH 7.2) was performed in a Spinco Model E analytical ultracentrifuge at 59,780 rpm at 25° C. A 4° single sector cell with a 12-mm aluminum centerpiece was used.

9. Digestion with bacterial hyaluronidases. There are several reasons for using bacterial rather than testicular hyaluronidases. 1) The bacterial enzymes used digest hyaluronate but not chondroitin sulfate. 2) The product of digestion is a Δ-4,5 unsaturated disaccharide (16) that is completely dialyzable. 3) Digestion is carried out at pH 6 to 7. On the other hand, digestion with testicular hyaluronidase must be carried out at pH 5 or lower to produce 100 per cent dialyzable fragments (1). Although tetrasaccharides are the main product, oligosaccharides are also produced that may dialyze with more difficulty.

Exhaustive digestion of HP with bacterial hyaluronidases was carried out as follows. A solution of HP (about 5 mg HP per g) previously dialyzed in a NaClacetate buffer at pH 6.2 was incubated with 1 mg of pneumococcal hyaluronidase (100 turbidity reducing units [TRU] per mg) for 16 hours at 37° C. Then 1 mg of streptococcal hyaluronidase (15 to 50 TRU per mg) was added and incubation carried out an additional 3 to 4 hours. More prolonged incubation was avoided because a thread-like precipitate formed; the explanation of this is not known. The digested product was dialyzed at 5° C in 500 ml of the NaClacetate buffer at pH 6.2, changed twice daily for 2 days. The hyaluronate fragments dialyzed completely; paper chromatography of a sample of the nondialyzable residue revealed no spots detectable with ultraviolet light or with aniline that would indicate the presence of the unsaturated disaccharide. Part of the nondialyzable residue was dialyzed in a NaClacetate buffer at pH 6.8 prior to further digestion with a liver enzyme containing β-glucuronidase and β-glucosaminidase.

10. Reaction with hydrazine (17). To 20 g of a solution of HP (1.5 mg HP per g) was added 20 ml of hydrazine (2 M) adjusted to pH 8.6 with glacial acetic acid. The mixture was incubated in a water bath at 40° C for 5 hours. Then 4 N HCl was added to bring the mixture to pH 5, and this solution was dialyzed at 5° C for 72 hours in 500 ml distilled water changed twice daily. The dialyzed solution was concentrated to about 3 ml and tested for free hydrazine and bound hydrazide, as follows.

a) Free hydrazine gives an immediate, intense color with Ehrlich's reagent (2g p-dimethylaminobenzaldehyde, 100 ml absolute ethanol, 30 ml concentrated HCl). To 1 g of the solution of the product in a 15 × 150 mm Pyrex tube 4 ml of Ehrlich's reagent was added, and the absorbance was read at once at 450 mµ in a Coleman, Jr. spectrophotometer. Run simultaneously were a reagent blank of 1 ml distilled water, a solution of HP not treated with hydrazine, and a standard, glutamyl hydrazide (0.02 µmole per ml). The absorption experiment was done as free hydrazine had been removed completely by dialysis.

b) Bound hydrazide. Each tube was covered with a glass marble and heated in a water bath at 80° C for 30 minutes. Bound hydrazides were hydrolyzed by this procedure, and the hydrazine liberated reacted with Ehrlich's reagent. The tubes were cooled, and the absorbance at 450 mµ was determined.

HP labeled with 14C was also treated with hydrazine, and zone electrophoresis was carried out at pH 4.5 in an acetate buffer (0.2 M). For controls, untreated 14C-labeled HP and 14C-labeled HP with added barbital buffer (0.1 M) at pH 8.6 were used.

11. Analysis. The methods for glucosamine and nitrogen have been described elsewhere (1). Protein was determined by a modified Folin method (18) with a γ-globulin solution as a standard. The nitrogen content of the protein standard was determined by a micro-Kjeldahl method. The hexuronic acid method of Dische (19) was used with modifications (20). The carbazole was twice recrystallized from alcohol and sublimed before use; after it was added, the tubes were allowed to stand for 2 hours and 45 minutes and the absorbance was determined in a Beckman DU spectrophotometer at 530 mµ. Ash was determined as K2SO4 after digestion with H2SO4, moisture by drying to constant weight at 105° C.

RESULTS

1. Isolation of HP by ultrafiltration. Data on the isolation of HP from pooled normal human synovial fluids are shown in Table I. The recovery of HP at each step is better than 90 per cent. The total yield of HP is about 75 per cent owing to some losses and to removal of samples at each step for analysis. After adsorption and two ultrafiltrations, little or no protein was removed by additional ultrafiltrations of HP.
 Adsorption on Ultrafiltration
3 Ultrafiltration were Progressive purification protein and tions were due processes should be used, and the number of times to use them, were studied. Table II shows that the molar ratios of nitrogen to glucosamine of the final products were similar whether 3 or 4 ultrafiltrations were followed by adsorption, or whether adsorption preceded ultrafiltration.

2. Iodination of HP with I^{131}. A method was needed to show the firm combination of all the protein and hyaluronate in the final product. One way to show this is moving boundary electrophoresis, but the protein content of HP was too low to permit detection by optical methods. It was necessary to label the protein with I^{131}. Then, by zone electrophoresis at pH 7.4 or 8.6, it was possible to compare the mobility of hyaluronate, by hexuronic acid, and protein, by radioactivity. The first point to establish was that I^{131} labeled only the protein and not the hyaluronate. This was shown in the following experiments. HP was labeled with I^{131} and then digested with streptococcal hyaluronidase. A part of this sample was dialyzed and about 95 per cent of the hyalu-

**TABLE I**

Data on the recovery of HP isolated from pooled normal human synovial fluids (22 g)

<table>
<thead>
<tr>
<th>Added</th>
<th>Recovered</th>
<th>Ratio mg protein/mg hyal.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hexur. acid</td>
<td>Protein</td>
</tr>
<tr>
<td>Adsorption on</td>
<td></td>
<td></td>
</tr>
<tr>
<td>column (IRC, hydroxylap.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ultrafiltration * 1</td>
<td>25.3</td>
<td>482</td>
</tr>
<tr>
<td>Ultrafiltration 2</td>
<td>22.6</td>
<td>30.3</td>
</tr>
<tr>
<td>Ultrafiltration 3</td>
<td>22.7</td>
<td>2.13</td>
</tr>
<tr>
<td>Ultrafiltration 3</td>
<td>20.7</td>
<td>1.08</td>
</tr>
</tbody>
</table>

* Ultracentrifugation after each ultrafiltration.

The order in which these purification procedures should be used, and the number of times to use them, were studied. Table II shows that the molar ratios of nitrogen to glucosamine of the final products were similar whether 3 or 4 ultrafiltrations were followed by adsorption, or whether adsorption preceded ultrafiltration.

**TABLE II**

Progressive purification of HP: results of the order and frequency of the procedures used

<table>
<thead>
<tr>
<th>Product</th>
<th>Order of procedure*</th>
<th>Ratio mg protein/mg hyal.</th>
<th>Molar ratio N/glucosamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1. Ultrafiltr. X3</td>
<td>.055</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2. Adsorpt. X1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3. Ultrafiltr. X1</td>
<td>.016</td>
<td>1.15</td>
</tr>
<tr>
<td>2b</td>
<td>1. Zone electroph. pH 5.4</td>
<td>.055</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2. Adsorpt. X1</td>
<td>.017</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1. Adsorpt. X1</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2. Ultrafiltr. X2</td>
<td>.013</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3. Adsorpt. X2</td>
<td>.018</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4. Ultrafiltr. X3</td>
<td>.013</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1. Ultrafiltr. X3</td>
<td>.036</td>
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</tr>
<tr>
<td></td>
<td>2. Adsorpt. X1</td>
<td>.022</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1. Adsorpt. X1</td>
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</tr>
<tr>
<td></td>
<td>2. Ultrafiltr. X2</td>
<td>.016</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3. Adsorpt. X1</td>
<td>.016</td>
<td>1.15</td>
</tr>
<tr>
<td>6</td>
<td>1. Adsorpt. X1</td>
<td>3.47</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2. Ultrafiltr. X3</td>
<td>.012</td>
<td>1.01</td>
</tr>
</tbody>
</table>

* After each ultrafiltration, the product was ultracentrifuged.

**FIG. 1. PROGRESSIVE PURIFICATION OF HP.** Zone electrophoresis at pH 7.4 on blocks of polyvinyl chloride of the following products obtained from synovial fluid: A, after 1 adsorption and 1 ultrafiltration, about 40 per cent protein; B, after 1 adsorption and 2 ultrafiltrations, 5 per cent protein; and C, final product (HP), after 1 adsorption and 4 ultrafiltrations, 2 per cent protein. The isolated products were labeled with I^{131} at different times, and the specific activities of the protein in A, B, C were different.
ISOLATION OF HYALURONATE PROTEIN FROM HUMAN SYNOVIAL FLUID

TABLE III

Analysis of HP *

<table>
<thead>
<tr>
<th>Product</th>
<th>Hexuronic acid</th>
<th>Glucosamine</th>
<th>Acetyl</th>
<th>Nitrogen</th>
<th>Protein†</th>
<th>Protein‡</th>
<th>Ash (K)</th>
<th>Moisture</th>
<th>N/GM‡</th>
<th>Hex. acid/ GM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>44.60</td>
<td>41.15</td>
<td>13.79</td>
<td>3.22</td>
<td>0</td>
<td>8.97</td>
<td>4.14</td>
<td>1.00</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>2a</td>
<td>34.82</td>
<td>31.91</td>
<td>2.87</td>
<td>1.88</td>
<td>2.31</td>
<td>8.39</td>
<td>6.41</td>
<td>1.15</td>
<td>1.01</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>32.86</td>
<td>28.83</td>
<td>2.61</td>
<td>1.75</td>
<td>2.25</td>
<td>8.16</td>
<td>1.82</td>
<td>1.16</td>
<td>1.05</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>36.90</td>
<td>34.23</td>
<td>1.31</td>
<td>2.50</td>
<td></td>
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<tr>
<td></td>
<td>39.30</td>
<td>34.94</td>
<td>3.87</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

* After dialysis in KAc, HP was precipitated with 3 volumes of absolute alcohol, washed with ether, and dried in vacuum.
† Folin method using a γ globulin standard.
‡ Calculated: total N − theoretical N (% glucosamine × 7.82) × 6.25.
§ GM—glucosamine.

Folinic acid fragments, but less than 3 per cent of the radioactivity, was recovered in the dialysate. The remainder of the digested I₁³¹-labeled sample was subjected to zone electrophoresis at pH 8.6. The hyaluronate fragments and the radioactivity appeared to be almost completely dissociated (see Figure 7,B).

To test whether some protein might be present

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**Figure 2. Acid (6 N HCl) hydrolysis and two-dimensional chromatography of HP.** 1, 2, 3-indantrione hydrate (Ninhydrin) spray. 1, leucine-isoleucine; 2, phenylalanine; 3, valine; 4, tyrosine; 5, alanine; 6, threonine; 7, glycine; 8, serine; 9, glutamic acid; 10, aspartic acid 11, arginine; 12, lysine; X, unidentified; GM, glucosamine.
in the isolated HP that I^{131} failed to label, a product was labeled with Cr^{51}Cl. Electrophoresis showed that almost 100 per cent of the radioactive material (Cr^{51}) migrated exactly with the hyaluronate toward the anode.

The progressive purification of HP during isolation from synovial fluid could be followed by zone electrophoresis of I^{131}-labeled products (Figure 1). After adsorption on the column followed by one ultrafiltration, electrophoresis of a labeled product showed that 90 per cent of the radioactivity remained at the origin or migrated more slowly than hyaluronate (Figure 1,A). With progressive purification of HP, an increasing proportion of the total radioactivity migrated exactly with hyaluronate (Figure 1,B). In the final products, over 90 per cent of the radioactivity migrated with hyaluronate as a single peak toward the anode (Figure 1,C).

3. Analysis of HP. The results of analyses of several products are shown in Table III. The molar ratio of hexuronic acid to glucosamine was 1.00 to 1.04. In three samples the molar ratio of nitrogen to glucosamine was 1.15, which is consistent with the presence of about 2 per cent protein. In one sample (no. 6) this ratio was 1.01, which would suggest that this product was protein-free; yet I^{131} labeled it, and acid hydrolysis and chromatography revealed amino acids.

The amino acids present in highest proportion in every product were serine, glycine, glutamic acid, and alanine (Figure 2). Smaller amounts of leucine, isoleucine, phenylalanine, valine, tyrosine, threonine, aspartic acid, and proline were identified. No hydroxyproline was present. Very faint traces of lysine and arginine were found. The only amino sugar observed was glucosamine; no neutral sugars were found. Many attempts to demonstrate hydrolysis products of nucleotides were unsuccessful, except on one occasion. Traces of uridine diphosphate glucosamine were found in one sample, and may have been due to bacterial contamination.

4. Viscosity. The viscosities of the starting synovial fluid and HP isolated from it by ultrafiltration were determined at several low concentrations of hyaluronate (.002 to .02 g per cent) in viscometers of the same velocity gradient. Extrapolation of a plot of the results to zero concentration of hyaluronate showed that the intrinsic viscosity of the synovial fluid and the isolated HP were similar (Figure 3). This indicated that very little degradation of HP occurred during its isolation. Figures of about 36 for intrinsic viscosity are among the highest reported for isolated hyaluronate. HP from a subject 21 years of age and from one 79 years of age showed the same intrinsic viscosities. Figure 4 shows the non-Newtonian or anomalous viscosity of HP. The high viscosity at very low shear rates fell abruptly as the shear rate was increased.

5. Isolation of HP by zone electrophoresis. Hyaluronate was also isolated from synovial fluid by zone electrophoresis at pH 5.4. The protein content of this isolated hyaluronate was about 6 per cent (Table II, 2b). A part of this sample was labeled with I^{131}. Zone electrophoresis of this labeled product at pH 8.6 showed that over 50 per cent of the protein migrated more slowly than hyaluronate, either moving as γ globulin or remaining at the origin. It was concluded that hy-
ISOLATION OF HYALURONATE PROTEIN FROM HUMAN SYNOVIAL FLUID

7. Isolation of hyaluronate from human synovial fluid

8. Isolation of hyaluronate from human synovial fluid

9. Isolation of hyaluronate from human synovial fluid

aluronate isolated by electrophoresis at pH 5.4 contained some γ globulin (9) which was bound to the hyaluronate by salt linkages and which was readily dissociated from hyaluronate by electrophoresis at pH 8.6. This extraneous protein was removed when the product isolated at pH 5.4 was adsorbed with hydroxylapatite. The protein content of this adsorbed product was 1.5 per cent. It was labeled with I131, and zone electrophoresis at pH 7.4 showed that over 90 per cent of the protein migrated exactly with hyaluronate. Like those products isolated by ultrafiltration and adsorption, the results of acid hydrolysis and paper chromatography of this product showed glucosamine, and predominantly serine, glycine, alanine, and glutamic acid.

6. Further evidence for a compound. a) Electrophoresis. I131-labeled HP migrated as a single peak toward the anode over a pH range from 11.2 to 3.5 (Figure 5). At pH 3.0 the product failed to move from the origin. Dialysis at pH 2.5 or 1.5 for 16 hours followed by zone electrophoresis at pH 4.5 showed a single peak migrating toward the anode. At pH 12.5 electrophoresis showed dissociation of hyaluronate and protein.

After electrophoresis at pH 7.4, I131-labeled HP was isolated from the block. The specific activity of this product was determined, and it was then subjected to electrophoresis over a pH range of 11.1 to 4.5. No change was found in the specific activity of the HP isolated after electrophoresis at each pH.

High salt concentration (0.3 M) or urea (6 M) did not dissociate HP. To test whether added protein could dissociate HP, the following was done. Lysozyme, used because its net charge is positive at neutral pH (21), was labeled with I131 and added to unlabeled HP (1.5 μEq hyaluronate, .03 μEq lysozyme I131). Zone electrophoresis at
pH 7.4 and 4.5 showed that lysozyme I^{131} migrated toward the cathode while HP moved toward the anode. In another experiment, lysozyme was added to I^{131}-labeled HP (0.3 μEq of each). HP migrated as a single component and no protein, as marked by radioactivity, was displaced from HP by lysozyme.

b) Ultracentrifugation. HP was sedimented as a single component during ultracentrifugation (Figure 6). Schlieren diagrams revealed one sharp symmetrical peak. The sharpness of the peak is apparently due to the high molecular weight of HP, and its symmetry suggests that HP is free of contamination.

c) Effects of digestion with bacterial hyaluronidases. HP was digested exhaustively with streptococcal and pneumococcal hyaluronidases, and then dialyzed. About 6 to 7 per cent of the total hexuronic acid remained in the nondialyzable residue. This finding suggests that part of the hyalu-
ronate is bound to protein through linkages resistant to digestion by hyaluronidase. Good evidence that the nondialyzable hexuronic acid was part of hyaluronate is shown in the following experiments, which are summarized here and in Figure 7.

Part of the nondialyzable residue that remained after hyaluronidase digestion was hydrolyzed in acid, and paper chromatography was carried out. The only amino sugar found was glucosamine. No galactosamine or neutral sugars were observed, indicating that no other anionic polysaccharide remained except hyaluronate residues. The remainder of the nondialyzable material was further digested with a liver enzyme containing β-glucuronidase and β-glucosaminidase, and dialyzed. Unfortunately, the presence of enzyme in both the dialysate and the nondialyzable material interfered with the colorimetric detection of hexuronic acid. It could be shown, however, that acetylglucosamine and hexuronic acid groups of hyaluronate in the nondialyzable residue were digested and rendered dialyzable by the liver enzyme. 1) Acetylglucosamine was detected in the dialysate by paper chromatography. 2) Electrophoresis at pH 4.5 of an 131-I labeled product digested with streptococcal hyaluronidase alone showed migration of about half the protein toward the anode (Figure 7,C), but after subsequent digestion with liver enzyme, almost all the protein remained immobile at the origin (Figure 7,D). This suggests removal by liver enzyme of hexuronic acid residues.

d) Effect of hydrazine. HP was treated with hydrazine under mild conditions that rupture ester bonds but avoid cleavage of peptide bonds. Free hydrazine was removed by dialysis, and bound hydrazides were measured by the intense yellow color formed after heating in the presence of HCl and p-dimethylanisobenzaldehyde. About 1.3 μmoles of bound hydrazide were formed per 1,000 mg of HP. Samples of HP not treated with hydrazine showed no color change after heating. The hydrazine-treated HP was digested with streptococcal hyaluronidase, and then dialyzed. The nondialyzable residue was tested for hexuronic acid, and about 8 per cent was found. A similar quantity of nondialyzable hexuronic acid had been found after streptococcal hyaluronidase digestion of HP without prior hydrazine treatment. This suggests that this nondialyzable hyaluronate residue is still bound to protein by bonds not susceptible to hydrazine treatment or streptococcal hyaluronidase digestion.

I131-labeled HP was also treated with hydrazine. Less than 10 per cent of the radioactivity was dialyzable. Zone electrophoresis at pH 4.5 (Figure 8,C) showed at least two effects when compared to controls: 1) most of the protein had been split from HP and remained near the origin, and 2) a small part of the protein migrated rapidly toward the anode in the hyaluronate zone. This suggests that some protein may still be bound to hyaluronate by bonds resistant to hydrazine treatment.

e) Effect of reducing agents. Ascorbic acid or cysteine reduces by about one-third the relative viscosity of HP solutions. Zone electrophoresis

![Figure 8](attachment:image.png)

**FIG. 8.** Treatment of 131-I labeled HP with hydrazine. Zone electrophoresis at pH 4.5. A, control, untreated. B, after incubation in barbital (0.1 M, pH 8.6) 5 hours at 40° C. HP moves as a single peak toward anode. Slight increase in protein at origin. C, after treatment with hydrazine (pH 8.6) 5 hours at 40° C. Large amount of protein split from HP and remains near origin. Part of protein still in hyaluronate zone.
TRINSIC VISCOSITY

The most of the domain peak toward the anode. If the protein were contained simply by mechanical entrapment within the domain of viscous hyaluronate, the lowering of hyaluronate's viscosity by reducing agents should have permitted the protein to be separated electrophoretically.

7. Effect of proteolytic enzymes. Either trypsin or papain, containing .04 M cysteine, caused proteolysis of the protein moiety of HP. Papain digestion of I\(^{131}\)-labeled HP caused about 40 percent of the radioactivity, but no hyaluronate, to become dialyzable. Zone electrophoresis showed that the protein fragments split into several peaks, and most of these fragments migrated more slowly than hyaluronate (Figure 9,B). Neither trypsin nor papain appreciably lowered the intrinsic viscosity of HP (Figure 10).

DISCUSSION

This paper describes mild methods to isolate hyaluronate from large volumes of pooled normal human synovial fluid. Hyaluronate containing about 2 per cent protein was isolated by a combination of adsorption, ultrafiltration, and ultracentrifugation. This product was called hyaluronate protein (HP). These isolation procedures were sufficiently mild so that the final product had an intrinsic viscosity similar to the starting synovial fluid, and possessed anomalous viscosity.

Hyaluronate can be separated from the bulk of the proteins of synovial fluid by a number of mild methods, such as electrophoresis, ultrafiltration, and precipitation with cetylpyridinium chloride, but additional procedures are always necessary to remove traces of contaminating protein (22). Proteolytic enzymes, such as pepsin, papain, pancreatic and intestinal extracts, and trypsin, have been used for this purpose. At least two of these enzymes, papain and trypsin, have been shown in the present study to remove most of the small quantity of protein firmly bound to hyaluronate. Contaminating protein has been removed by inert adsorbents such as Lloyd's reagent, Celite, and charcoal-cellulose mixtures, and by ultracentrifugation. By a combination of electrodereposition, adsorption, and ultracentrifugation, there have been isolated from synovial fluids some products apparently free of protein, since their molar ratios of nitrogen to glucosamine were 1.00 (3, 4). As this paper shows, however, the very small quantity of protein firmly bound to hyaluronate may not be detected by analyses for nitrogen and glucosamine. In the present study, protein was detected by showing the presence of amino acids, by labeling the protein with T\(^{131}\), and by demonstrating proteolysis after digestion with trypsin or papain. It was then necessary to show that hyaluronate and protein were combined. Perhaps the most suitable method, and one that appears not to have been previously applied for this purpose, is the iodination of the protein moiety and determination of the electrophoretic behavior of the labeled product. The evidence presented in this paper that HP is a compound may be summarized as follows.

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**FIG. 9. EFFECT OF PAPAIN ON I\(^{131}\)-LABELLED HP.** Zone electrophoresis at pH 8.6. A, control treated with cysteine (0.04 M) alone. B, treated with papain + cysteine.

**FIG. 10. EFFECT OF PROTEOLYTIC ENZYMES ON INTRINSIC VISCOSITY OF HP.** HP alone (●); after trypsin (●); and after papain (○).
1. Repeated attempts to remove additional protein by adsorption of HP—with Celite, carboxymethyl cellulose, or IRC-50 and hydroxylapatite—failed to lower the protein content.

2. Products isolated after multiple ultrafiltrations and adsorptions showed similar analyses.

3. A product obtained by an entirely independent method, zone electrophoresis at pH 5.4 and adsorption, showed a similar protein content and similar amino acid composition to HP isolated by ultrafiltration.

4. Acid hydrolysis and paper chromatography of each isolated product showed the same amino acids in high proportion: alanine, serine, glycine, and glutamic acid.

5. The I\textsuperscript{131}-labeled protein and hyaluronate migrated as a single peak over a pH range of 11.2 to 3.5. HP was not dissociated by low pH (1.5), high salt concentration (0.3 M), or urea (6 M). Cysteine or ascorbic acid, which markedly reduced the viscosity of HP, did not dissociate it. Nor did lysozyme, a protein with a net cationic charge at neutral pH, dissociate HP or displace the protein moiety of HP.

6. After exhaustive digestion of HP with bacterial hyaluronidase and dialysis, there remained a nondialyzable fraction containing all the protein and 6 to 7 per cent of the hyaluronate. This is good evidence that hyaluronate is bound to protein by chemical bonds not susceptible to bacterial hyaluronidase digestion. Further digestion of the nondialyzable residue with a liver enzyme containing \( \beta \)-glucuronidase and \( \beta \)-glucosaminidase removed hexuronic acid and acetylglucosamine groups of hyaluronate.

Dissociation of HP at pH 12.5 suggested that hyaluronate and protein might be combined by ester linkages. To study this possibility, HP was treated with hydrazine. This reagent has been used to demonstrate “ester-like” linkages in collagen (23). Under the mild conditions employed in the present study, ester bonds should be cleaved with formation of hydrazides at the COO\(^-\) position where the ester was previously present. Alternatively, a hydrazone might be formed at the reducing end of the hyaluronate. It was found that 1.3 \( \mu \)moles of bound hydrazide or hydrazone were formed per 1000 mg of HP. Expressed as moles, 1.3 moles of bound hydrazide or hydrazone were formed per \( 10^6 \) g of HP. This result is compatible with formation of a hydrazone, assuming the molecular weight of hyaluronate to be \( 10^6 \). On the other hand, there is the possibility, based on the results of electrophoresis of hydrazine-treated, I\textsuperscript{131}-labeled HP, and on the probability that the molecular weight of hyaluronate exceeds \( 10^6 \) (4), that ester bonds might have been broken and a hydrazide formed. In an attempt to resolve this question, hydrazine-treated HP was digested with streptococcal hyaluronidase and dialyzed. Bound hydrazides were found in both the dialysate and the nondialyzable fractions, suggesting that ester bonds were split, but the results must still be considered tentative because of color imparted to the solutions by the enzyme. If hydrazine treatment broke ester bonds, such bonds might be formed 1) through COO\(^-\) groups of hyaluronate and OH groups of serine or threonine, 2) through COO\(^-\) groups of dicarboxylic amino acids and OH groups of hyaluronate, or 3) through terminal COO\(^-\) groups of an amino acid and OH groups of hyaluronate; an example of this type of bond in a protein polysaccharide is found in teichoic acids where terminal COO\(^-\) groups of alanine residues are linked to OH groups (either glucosyl or ribitol) of the polymer (24). In addition to these three possibilities, inter-chain ester links could be present in either hyaluronate or protein.

Assuming that hydrazine-treatment of HP led to rupture of ester bonds rather than hydrazone formation, a preliminary picture may be proposed for the structure of HP (Table IV). This scheme is based chiefly on: 1) the finding of 6 to 7 per cent of nondialyzable hyaluronate after digestion of HP, or of hydrazine-treated HP, with streptococcal hyaluronidase; 2) the failure of proteolytic enzymes to reduce the intrinsic viscosity of HP; and 3) the electrophoretic pattern observed after hydrazine-treatment of I\textsuperscript{131}-labeled HP (Figure 8C).

Comparison of HP with the more widely studied chondromucoprotein (CMP) isolated chiefly from bovine nasal cartilage is relevant (Table IV). Unlike HP, preparations of CMP contain about 25 per cent protein firmly bound to chondroitin sulfate (25–28). Like HP, the protein of CMP contains a high proportion of dicarboxylic amino acids and serine, and Muir (25) suggested that serine of the protein might be combined in ester-linkage with part of the “acidic groups” of chon-
TABLE IV
Comparisons of HP and chondromucoprotein (CMP) from cartilage.

<table>
<thead>
<tr>
<th></th>
<th>HP</th>
<th>CMP</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>% protein</td>
<td>2</td>
<td>About 25</td>
<td>(25-28)</td>
</tr>
<tr>
<td>Intrinsic viscosity</td>
<td>35</td>
<td>2.7 - 4.5</td>
<td>(26,27)</td>
</tr>
<tr>
<td>Viscosity after proteolytic ens.</td>
<td>Little change</td>
<td>Marked fall</td>
<td>(25)</td>
</tr>
<tr>
<td>Effect of alkali</td>
<td>Dissociation</td>
<td>Dissociation</td>
<td>(25,26,29)</td>
</tr>
<tr>
<td>Other anionic polysac.</td>
<td>None/None found</td>
<td>? Keratosulfate</td>
<td>(28)</td>
</tr>
<tr>
<td>Major amino acids in protein</td>
<td>Dicarboxylic; serine; glycine; alanine</td>
<td>Same</td>
<td>(25,27)</td>
</tr>
<tr>
<td>Ultracentrifugation</td>
<td>1 component</td>
<td>2 components</td>
<td>(26)</td>
</tr>
<tr>
<td>Possible structure</td>
<td></td>
<td></td>
<td>(27,29)</td>
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J. Sandson and D. Hamerman

droitin sulfate. The links between protein and chondroitin sulfate are disrupted by prolonged incubation in alkali (26, 29). The effects of proteolytic enzymes point out major differences in the structure of CMP and HP. The moderately high viscosity of solutions of CMP is markedly reduced after digestion with trypsin or papain, and the molecular weight falls from 10^6 or more to less than 50,000. This finding has led to suggestions that many chondroitin sulfate units of short chain length are bound to a long protein core. Hyaluronate chains are probably much longer and occupy a domain that is even larger than their actual size (30). Interpenetration or overlap of these highly solvated molecules, even at low concentration, accounts for the extremely high viscosity and shear-dependent viscosity of hyaluronate solutions. Digestion of HP with proteolytic enzymes does not reduce appreciably the intrinsic viscosity or the anomalous viscosity (6) of hyaluronate solutions. This suggests that hyaluronate exists as an uninterrupted long-chain polymer with protein present as side chains, or at the end(s) of the hyaluronate molecule, or both.

The biological role of the small amount of protein firmly bound to the hyaluronate is not known. The protein may play some role, not yet defined, in the physical properties of the hyaluronate molecule; it may participate in the synthesis of hyaluronate in synovial membrane cells, or protein may be added to the hyaluronate at the cell wall, and play a role in the passage of hyaluronate into the synovial fluid.

SUMMARY

1. From normal human synovial fluids, hyaluronate consistently containing about 2 per cent protein was isolated by a combination of ultrafiltration and adsorption. A similar product was
obtained from synovial fluids by zone electrophoresis at pH 5.4 and adsorption. Evidence that hyaluronate and protein were firmly combined led to use of the term hyaluronateprotein (HP) to describe this product.

2. HP was labeled with I\(^{131}\), and zone electrophoresis over a pH range of 11.2 to 3.5 showed migration of hyaluronate, measured as hexuronic acid, and protein, measured by radioactivity, as a single peak toward the anode. Neither high salt concentration, urea, nor addition of a basic protein dissociated hyaluronate and protein.

3. After exhaustive digestion of HP with bacterial hyaluronidase and dialysis, there remained about 6 to 7 per cent nondialyzable hyaluronate bound to all the protein. This is good evidence that hyaluronate is bound to protein by chemical bonds not susceptible to bacterial hyaluronidase digestion. Further digestion of the nondialyzable residue with a liver enzyme containing \(\beta\)-glucuronidase and \(\beta\)-glucosaminidase removed hexuronic acid and acetylglucosamine groups of hyaluronate.

4. Treatment of HP with hydrazine led to formation of 1.3 \(\mu\)moles of nondialyzable hydrazide or hydrazone per 1000 mg of HP. Some evidence suggests that ester bonds were split and a hydrazide formed.

5. A preliminary scheme for the structure of HP is proposed.

ACKNOWLEDGMENT

We wish to thank Drs. Paul Gallop and Olga Blumenfeld for helpful discussions concerning the use of hydrazine.

ADDENDUM

The free aldehyde group of HP was reduced with NaBH\(_4\), and the product then treated with hydrazine. The same amount of bound hydrazide was formed as in control samples not reduced with NaBH\(_4\), indicating that hydrazides were formed and not a hydrazone.

REFERENCES


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