The Isolation of Minimally Degraded Hyaluronate from Rat Skin

By JOYCE M. MATHIESON and RICHARD H. PEARCE

*Department of Pathology, Faculty of Medicine, University of British Columbia, Vancouver, B.C. V6T 1W5, Canada, and
Institute of Medical and Physiological Chemistry, Biomedical Centre, University of Uppsala, Uppsala, Sweden

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The major macromolecular constituents of the 'ground substance' of rat skin and human dermis are plasma protein and hyaluronate (Sweeny et al., 1963; Mathieson & Pearce, 1963; Pearce & Grummer, 1972). The dermatan sulphate of the dermis appears to be bound to the insoluble collagen (Pearce & Grimmer, 1972; Obrink, 1973).

The present study describes the quantitative isolation of hyaluronate from fresh rat skin and compares the properties of the material with umbilical-cord hyaluronate.

Materials and Methods

Chemicals

Reference hyaluronate was prepared from human umbilical cord (Alburn & Williams, 1950); reference chondroitin 4-sulphate was purchased from Nutritional Biochemicals Corp., Cleveland, OH, U.S.A., and purified by further treatment with NaOH and phenol (Pearce & Mathieson, 1967); dermatan sulphate (ox lung b-heparin) was a gift from Professor L. B. Jaques, Department of Physiology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, and was purified further as described by Jeanloz (1965). All other chemicals were commercial reagent grade.

Analytical methods

Hexuronate was measured as described by Bitter & Muir (1962) by using sodium glucuronate monohydrate (Corn Products Refining Co., New York, NY, U.S.A.) as a standard. Hexosamine was measured as described by Roseman & Daffner (1956) with a glucosamine hydrochloride standard, omitting the use of a resin column and prolonging the hydrolysis time to 16h in vacuo. The hexosamines were identified by descending paper chromatography (Fischer & Nebel, 1955) with glucosamine and galactosamine standards, followed by silver staining [the recommended method of Block et al. (1958), modified by substitution of dipping in 0.08 M NaOH in ethanol for spraying with NaOH]. Nitrogen was measured by the micro Kjeldahl nesslerization procedure of King (1951a,b); NaN₃ was not detected by this procedure. Protein was detected by its A₄₅₀ by using a 10mm cell in a Beckman DU spectrophotometer. Electrophoresis on cellulose acetate (Sepraphore III, Gelman Instrument Co., Ann Arbor, MI, U.S.A.) used 0.15m-zinc acetate (Haruki & Kirk, 1967) followed by staining with Alcian Blue (Foster & Pearce, 1961). Chloride was titrated with Hg(NO₃)₂, with 's-diphenyl-carbazone' (Eastman Organic Chemicals, Rochester, NY, U.S.A.) to detect the end point, as described earlier (Pearce et al., 1968).

Physical methods

Solutions were concentrated by pressure ultrafiltration (model no. 52 or 202 cells; Amicon, Lexington, MA, U.S.A.) by using UM10 or PM10 membranes (Amicon). Viscosity was measured in a Cannon–Manning semi-micro viscometer, size 100 (Cannon Instrument Co., State College, PA, U.S.A.) in a water bath maintained at 25.0 ± 0.1°C. Three to five dilutions, prepared gravimetrically, were measured. The hyaluronate content of the sample after dilution was measured as hexuronate and calculated assuming a hexuronate content of 2.5μmol/mg. The reduced viscosity for three or more points was extrapolated to infinite dilution by using a calculated line of best fit (Snedecor, 1946).

Specimen

Male Sprague–Dawley rats weighing 120–280g were used. Immediately after being killed with an overdose of diethyl ether vapour, the rats were
shaved and skinned carefully to exclude subcutaneous fascia, adipose tissue and muscle. The skin was minced with scissors and either ground in a chilled Latapie mill (A. H. Thomas Co., Philadelphia, PA, U.S.A.), or homogenized in a bacterial press (Edebo, 1960) after cooling at −50°C for 30 min.

**Saline extracts**

The ground fresh skin was extracted with 0.10M-NaCl containing 0.05M-Tris/HCl, pH 7.5, and 0.2 mg of NaN₃/ml to inhibit bacterial growth. Two 2h treatments at 4°C, the first with 5 ml, the second 15 ml, per g fresh wt. were performed as described by Sweeney et al. (1963). The pooled extracts were clarified by vacuum filtration after the addition of Hyflo Supercel.

**Determination of glycosaminoglycans**

The glycosaminoglycans were isolated by a slight modification of the procedure of Pearce & Mathieson (1967): after digestion with papain, crude glycosaminoglycans were precipitated from the digest by the addition of 3 vol. of ethanol containing 1% (v/v) acetic acid and 1% (w/v) potassium acetate. After standing at 4°C overnight, the precipitate was collected by centrifugation at 10000g and 4°C for 10 min and washed twice with 75% (v/v) ethanol. After drying, the precipitate was dissolved in 0.01M-NaOH and its hexuronate content measured.

**Fractionation and characterization of the glycosaminoglycans**

Crude glycosaminoglycans, prepared as described above, were precipitated as their cetylpyridinium complexes, the glycosaminoglycans recovered and purified by treatment with NaOH, phenol and tri-chloroacetic acid (Pearce & Mathieson, 1967). The hyaluronate and monosulphated glycosaminoglycans were separated from the mixture by selective elution from AG-1X2(Cl⁻ form) [Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ont., Canada] micro-columns (2mm×10mm) with 0.75M-NaCl/8M-urea and 1.50M-NaCl/8M-urea respectively (Pearce et al., 1968, 1972). The recovery of reference hyaluronate was approx. 70%; that of the sulphated glycosaminoglycans was greater than 90%. Each fraction was analysed for hexuronate and characterized by electrophoresis.

**Ion-exchange chromatography**

DEAE-cellulose (Whatman DE-32 or DE-52; H. Reeve Angel Inc., Clifton, NJ, U.S.A.) was regenerated as recommended by the supplier and equilibrated with 0.05M-Tris/HCl, pH 7.5. Gradients were formed by feeding the column from two 400 ml beakers connected by an inverted glass U-tube.

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**Table 1. Effect of the methods of homogenization on the yield of hyaluronate**

<table>
<thead>
<tr>
<th>Skin pool no.</th>
<th>Edebo press</th>
<th>Latapie mill</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.76</td>
<td>0.26</td>
</tr>
<tr>
<td>B</td>
<td>0.69</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>0.70</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>0.69</td>
<td>0.29</td>
</tr>
</tbody>
</table>

Gel chromatography

A column (2.6cm×85cm) of Sephadex G-200 [Pharmacia (Canada) Ltd., Montreal, P.Q., Canada] equilibrated with 0.10M-NaCl containing 0.05M-sodium acetate, pH 5.5, and packed in the cold, was operated with a peristaltic pump, adjusted to operate at one-half the flow rate attained with a 15cm head. The collection of 12.5 ml fractions was timed.

**Results and Discussion**

**Saline extraction**

Most of the hyaluronate of freeze-dried fat-free rat and human skin could be extracted with 0.15M-NaCl (Mathieson & Pearce, 1963; Pearce & Grimmer, 1972). However, since freeze-drying is a first step in our original procedure and the process degrades hyaluronate (Jeanloz & Forchielli, 1950), such material could not be used for this work. Although saline extracts of fresh skin are difficult to clarify, probably because some adipose tissue remains even after careful dissection, Hyflo Supercel may be used with little loss, provided that the filter pad is washed well (Pearce & Mathieson, 1967). Because of the possible degradation of hyaluronate post mortem, a ‘rapid’ procedure was devised after re-examination of results published earlier (Sweeney et al., 1963); two extractions of 2h duration seemed likely to include almost all the hyaluronate extractable with isosmotic saline.

In contrast with the results with freeze-dried fat-free skin, 50–75% of the cutaneous hyaluronate remained unextracted with buffered saline after mincing fresh skin with a Latapie mill. Access to a bacterial press (Edebo, 1960), which had previously been used successfully to homogenize rat skin (Öbrink, 1972), suggested its comparison with the Latapie mill with possible improvement of the yield of hyaluronate.

The hyaluronate found in three portions of fresh skin after isolation and fractionation as described in...
RAT SKIN HYALURONATE

Saline extract (525ml), prepared from 20g of fresh skin, was dialysed against running tap water and applied to a 2.5cm x 5cm column of DEAE-cellulose DE-32 (A). The column was washed with 200ml of 0.05m-pyridine/HCl, pH5.5 (B), and then eluted with a gradient formed with 250ml of 0.05m-pyridine/HCl, pH5.5, in the proximal vessel and 250ml of the same buffer containing 1m-NaCl in the distal vessel (C); 15.4ml fractions were collected during the application and wash, and 6.3ml during the gradient. All fractions were analysed for hexuronate (—) and A_{280} (· · ·), every fifth fraction in the gradient, for chloride (○).

**Table 2. Analysis of hyaluronate preparations**

Three separate pools of rat skin were used to prepare hyaluronate by homogenization in an Edebo press, extraction with Tris-buffered NaCl, fractionation on DEAE-cellulose and chromatography on Sephadex G-200. After concentration by pressure-ultrafiltration and dialysis against 0.20m-NaCl containing 0.01m-sodium phosphate, pH7.4 (Cleland & Wang, 1970), the viscosity of one portion of each preparation was measured. Subsequently, this and a second portion were dialysed against distilled water, freeze-dried and analysed for hexuronate, hexosamine and nitrogen. Two portions of reference umbilical-cord hyaluronate were treated similarly for comparison. One portion of preparation no. 2 was lost. Further details are given in the Materials and Methods section.

<table>
<thead>
<tr>
<th>Preparation no.</th>
<th>Yield (μmol of hexuronate/g fresh wt.)</th>
<th>Molar ratios</th>
<th>[η] (litre/g of hyaluronate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat skin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.68</td>
<td>0.96</td>
<td>1.50</td>
</tr>
<tr>
<td>2</td>
<td>0.87</td>
<td>1.06</td>
<td>1.41</td>
</tr>
<tr>
<td>3</td>
<td>0.67</td>
<td>0.97</td>
<td>3.09</td>
</tr>
<tr>
<td>Human umbilical cord</td>
<td>—</td>
<td>1.01</td>
<td>3.29</td>
</tr>
</tbody>
</table>

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the Materials and Methods section, under conditions giving approx. 70% yield, was 0.52, 0.44 and 0.42 μmol of hexuronate/g fresh wt. Two additional pools of rat skin were divided; one portion of each was homogenized in the Edebo press, the second in the Latapie mill. Each lot of homogenized tissue was extracted with 0.10 M-NaCl containing 0.05 M-pyridine/HCl, pH 7.5, concentrated and the hyaluronate content measured (Table 1). Quantitative yields were obtained with the Edebo press, whereas the yield from material ground in the Latapie mill was about one-third (Table 1). Consequently, the press was used in subsequent experiments.

**Optimal conditions for ion-exchange chromatography**

DEAE-cellulose chromatography was a convenient method for the concentration and partial purification of hyaluronate from the saline extract; optimal conditions were examined in some detail. Saline extract was dialysed and applied to a column of DEAE-cellulose. The column was washed with 0.05 M-pyridine/HCl, pH 5.5, and then eluted with a NaCl gradient in the same buffer (Fig. 1). Although the hexuronate and most of the protein were bound when the extract was applied to the column, the wash at pH 5.5 released a major protein peak. A second peak containing little hexuronate appeared near the beginning of the gradient. At 0.25 M-Cl⁻, a sharp hexuronate peak appeared with little protein. This had emerged almost completely by 0.40 M-Cl⁻, at which concentration a third peak containing protein and hexuronate appeared. The latter had been completely eluted by 0.75 M-Cl⁻. A second experiment gave a very similar elution pattern. On electrophoretic analysis, the major hexuronate peak (0.25–0.40 M-Cl⁻) resembled hyaluronate. The lesser hexuronate peak (0.40–0.75 M-Cl⁻) gave a diffuse band, of which the most intense part resembled dermatan sulphate; some suggestion of traces of hyaluronate was seen in the smeared pattern which extended back to the origin.

The data obtained from the gradient columns were used to devise a step-elution procedure (Fig. 2). Dialysis could be omitted, since the major hexuronate peak did not appear until 0.25 M-Cl⁻. Much of the protein did not bind to the ion-exchanger when the saline extract was applied directly; more was released when the column was washed with 0.25 M-Cl⁻ at pH 5.5, and 0.40 M-Cl⁻ eluted a sharp hexuronate peak and considerable protein. A small peak containing
Saline extract was prepared and clarified, and the extract was applied to a column (2 cm × 24.5 cm) of DEAE-cellulose (DE-32, Whatman) and washed with 1 litre of 0.20 M NaCl containing 0.05 M pyridine/HCl, pH 5.5, then with 1 litre of 0.35 M NaCl containing 0.05 M pyridine/HCl, pH 5.5. The latter was dialysed against water and concentrated to 12 ml. The concentrate was dialysed against 0.10 M NaCl containing 0.05 M sodium acetate, pH 5.5, overnight; the contents of the sac were centrifuged at 16000g for 20 min at 4°C, and the supernatant was applied to a column (2.6 cm × 85 cm) of Sephadex G-200 and eluted with 0.10 M NaCl containing 0.05 M sodium acetate, pH 5.5; 12.5 ml fractions were collected and analysed for hexuronate (——) and A_{280} (· · ·).
graphy of the hexosamine (Table 2). All data indicated that hyaluronate was the sole glycosaminoglycan present: the hexuronate/hexosamine molar ratio was 1:1 within the experimental error, glucosamine was the only hexosamine detected after paper chromatography and, on electrophoresis, a single spot with the mobility of a non-sulphated glycosaminoglycan was seen. The excess of nitrogen over hexosamine indicates the presence of protein in the preparation from rat skin. The intrinsic viscosities of all preparations were very similar and corresponded to an average molecular weight of approx. $3 \times 10^9$ (Cleland & Wang, 1970).

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References