THE METABOLISM OF MUCOPOLYSACCHARIDES IN ANIMALS

I. ISOLATION FROM SKIN*

BY SARA SCHILLER, MARTIN B. MATHEWS,† HAROLD JEFFERSON, JULIO LUDOWIEG, AND ALBERT DORFMAN

(From La Rabida Jackson Park Sanitarium, and the Departments of Pediatrics and Biochemistry, University of Chicago, Chicago, Illinois)

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Although the importance of the mucopolysaccharides of the ground substance of the connective tissue has been stressed by many biological and histochemical studies, little information is available regarding the metabolism of these substances. This can be attributed in part to difficulties in obtaining biological material appropriate to metabolic studies, from which adequate amounts of mucopolysaccharides can be isolated.

In 1941, Meyer and Chaffee (1) reported a method for the separation of both hyaluronic (HA) and chondroitinsulfuric acid (CSA) from fresh pig skin based upon alcohol fractionation of the barium salts. The method is inadequate, however, for the complete separation of the two substances. The recent publication by Gardell et al. (2) of a procedure for the separation of mucopolysaccharides by electrophoresis on a slab of Hyflo Super-Cel suggested that the isolation of these compounds in a high state of purity might be feasible.

This report is concerned with the development of methods for the isolation of the mucopolysaccharides of sufficient analytical purity from the skin of individual rabbits to permit metabolic studies. Subsequent reports will be concerned with metabolic studies in which these procedures have been employed.

EXPERIMENTAL

Preparation of Crude Extracts—All the animals used were normal adult male rabbits of the market variety maintained on standard Rockland rabbit chow. They were shaved and skinned immediately after death by air embolism. The fresh skin was freed of visible fat, rinsed with cold water, and ground twice in an electric meat grinder. It was defatted with acetone and allowed to dry at room temperature.

The dried skin was extracted with a 2 per cent solution of NaOH (15 ml. per gm. of dry skin) by shaking at room temperature for 24 hours in a

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† Established Investigator of the American Heart Association.
mechanical shaker. A small amount of thymol was added to prevent bacterial contamination. At the end of 24 hours the mixture was strained and the extract stored at 4°. The extraction was repeated with one-third the original volume of NaOH and the two extracts were pooled.

The combined extracts were dialyzed against running tap water for 48 hours and then against distilled water at 4° for 48 to 72 hours, at which point the pH of the mixture was 7.8 to 8.0. The extract was incubated with crystalline trypsin¹ (2.5 mg. for each gm. of protein) in cellophane casings and was dialyzed simultaneously against 0.1 M phosphate buffer, pH 7.8 to 8.0, at 37° for 5 days.

At the end of the digestion period, one-third volume of 40 per cent trichloroacetic acid was added slowly with constant stirring, and the mixture allowed to stand at least 30 minutes. After centrifuging, the clear supernatant fluid was dialyzed against distilled water at 4° for 72 hours. A precipitate which formed during dialysis was removed by centrifugation.

The dialyzed extract was concentrated on the steam bath to approximately a tenth the original volume, and the crude polysaccharides were precipitated by the addition of 4 volumes of 95 per cent ethanol in the presence of 1 per cent sodium acetate. After 3 days at 4°, the precipitate was collected by centrifugation, washed three times with 95 per cent ethanol and once with ether, and dried in a vacuum desiccator over paraffin.

The nitrogen and hexosamine content of the extract was followed throughout the stages of purification. The results of such analyses are illustrated in Table I. Hexosamine was determined by a modification of the method of Elson and Morgan (3).

Slab Electrophoresis of Crude Polysaccharides—Experiments with rat skin mucopolysaccharides (4) indicated that electrophoresis in a slab of Hyflo Super-Cel would separate the two fractions with but slight mutual contamination. The apparatus described by Gardell et al. (2) for the electrophoresis of the acid polysaccharides was modified, therefore, to accommodate 200 to 300 mg. quantities of the crude mixture. This would permit the isolation of sufficient material for adequate analyses.

The supporting medium consisted of Celite analytical filter aid (Johns-Mansville), moistened with a solution containing a mixture of 0.09 M sodium chloride and 0.01 M sodium phosphate buffer, pH 7.0, saturated with thymol as a preservative. The filter aid was packed in an acrylate plastic bed, 1 cm. deep, 10 cm. wide, and 25 cm. long, and the crude skin polysaccharide was added by replacing a section 1 cm. wide from the center of the slab with filter aid moistened with a buffer solution of 200 to 300 mg. of the preparation. A potential gradient of 2 volts per cm. with a current of 100 to 120 ma. was applied across the slab for 24 hours, and water at 30°

¹ The crystallized trypsin was kindly supplied by Armour and Company.
was circulated through a hollow acrylate box above and below the slab, to prevent heating. At the end of the 24 hour period the slab was divided into strips 1 cm. wide, which were eluted individually with 10 ml. of fresh buffer. The eluate from each strip was analyzed for uronic acid by the carbazole reaction of Dische (5), and the cuts corresponding to each peak were combined, dialyzed, precipitated, and dried as described previously.

Fig. 1 demonstrates the result of a typical electrophoresis pattern of the mucopolysaccharides from rabbit skin. It should be noted that, owing to extensive electroosmotic movement of the buffer itself, the HA fraction was displaced toward the cathode. In free solution, the fast component migrated about 60 per cent faster than the slow component (Fig. 2). If it is assumed (6) that the rates of migration in a stabilized electrolyte are proportional to the respective mobilities in free solution, it may be esti-

**Table I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Nitrogen</th>
<th>Hexosamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkali extract</td>
<td>154.0</td>
<td>2.7</td>
</tr>
<tr>
<td>Dialyzed extract</td>
<td>48.6</td>
<td>1.9</td>
</tr>
<tr>
<td>Supernatant following pptn. with trichloroacetic acid</td>
<td>0.88</td>
<td>1.1</td>
</tr>
<tr>
<td>Sodium salt of crude polysaccharide*</td>
<td>0.11</td>
<td>0.8</td>
</tr>
</tbody>
</table>

* The crude extract weighed 154.4 mg. and contained 3.6 per cent nitrogen and 25.0 per cent hexosamine.

mated that electroosmosis caused the buffer to be displaced about 26 cm. to the left of the origin. The net distance of movement, relative to the buffer, for the fast and the slow moving components was 32 and 20 cm., respectively. Thus substances of low electrical charge, such as glycogen and proteins, were separated from the acid polysaccharides.

Electrophoretic analysis of the two mucopolysaccharide fractions obtained by slab electrophoresis was carried out in a Perkin-Elmer Tiselius apparatus. The patterns indicated the presence of a single component in each of the isolated fractions (Fig. 2).

**Results**

**Analysis of Hyaluronic Acid and Chondroitinsulfuric Acid Fractions**—The results of the chemical analyses of the acid polysaccharides isolated from the skin of rabbits are presented in Table II. The absolute values were somewhat lower than theory, due probably to retention of moisture even after extreme drying (10). A high degree of purity for both the HA
and CSA fractions is demonstrated, nevertheless. On the basis of nitrogen as 1.00, ratios of 1.00, 0.96, and 1.02 for uronic acid, hexosamine, and acetyl of the HA were found. In a similar fashion for the CSA fraction, the ratios of uronic acid, hexosamine, acetyl, and sulfur were 0.94, 0.91, 0.94, and 0.96.

It should be noted that, while the uronic acid analysis of the CSA fraction by the manometric CO₂ method (8) approached theory, the analysis by the carbazole reaction (5) was only 37 per cent of theory.²

In neutral solution, the [α]₅₀ = -78° for the HA, while the [α]₅₀ = -55° for the CSA fraction of rabbit skin. These values agree essentially with those reported by Meyer and Rapport (12) for the corresponding fractions from pig and calf skins.

**Identification of Hexosamine of Hyaluronic Acid and Chondroitin Sulfuric Acid Fraction**—Samples of the HA and CSA fractions were each hydrolyzed for 8 hours in 4 N HCl. After treating with Norit A, the hydrolysates were concentrated in vacuo and the excess HCl was removed by the addition of water to the residue and repeated concentration under reduced pressure. The respective residues were taken up in 0.3 N HCl and chromatographed on a column of Dowex 50 according to the method of Garde11 (13). Analysis of the eluates for hexosamine by a modification of the method described by Tracey (14) demonstrated that the hydrolysates of the HA and CSA fractions each contained a single peak. The distribution of the hexosamine from the HA and CSA hydrolysates corresponded to

² Meyer (11) states that the CSA from pig skin differs considerably from cartilage CSA in the time-intensity curve of the reaction with carbazole.
that of crystalline glucosamine hydrochloride and chondrosamine hydrochloride respectively.

To facilitate the crystallization of the hexosamine from the hydrolysate of the CSA fraction, the appropriate eluates were combined, concentrated

\[ \text{in vacuo, and rechromatographed with 0.05 N HCl on a fresh column of Dowex 50.} \]

The fractions containing the amino sugar of the HA and the CSA were each combined, concentrated in vacuo, and the respective hexosamines crystallized from acetone. The x-ray diffraction pattern\(^8\) of the hexosamine

\[ \text{The x-ray diffraction patterns were prepared and analyzed by Dr. Fritz Laves of the University of Chicago.} \]
**TABLE II**

*Chemical Analyses of Mucopolysaccharide Fractions from Rabbit Skin*

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Loss on drying*</th>
<th>N†</th>
<th>S‡</th>
<th>Hexuronic acid§</th>
<th>Hexuronic acid∥</th>
<th>Hexosamine‡</th>
<th>N-Acetyl**</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RHA-1</td>
<td>8.5</td>
<td>3.18</td>
<td>&lt;0.1</td>
<td>48.4</td>
<td>48.4</td>
<td>39.0</td>
<td>10.00</td>
</tr>
<tr>
<td>Theory, %</td>
<td></td>
<td>91.1</td>
<td></td>
<td>90.5</td>
<td>96.3</td>
<td>87.2</td>
<td>93.2</td>
</tr>
<tr>
<td>CSA</td>
<td></td>
<td>0</td>
<td>2.78</td>
<td>38.6</td>
<td>38.6</td>
<td>35.6</td>
<td>8.55</td>
</tr>
<tr>
<td>RCSA-1</td>
<td>11.3</td>
<td>2.75</td>
<td>6.03</td>
<td>35.7</td>
<td>14.3</td>
<td>32.1</td>
<td>7.92</td>
</tr>
<tr>
<td>Theory, %</td>
<td></td>
<td>98.9</td>
<td>94.7</td>
<td>92.5</td>
<td>37.0</td>
<td>90.2</td>
<td>92.7</td>
</tr>
</tbody>
</table>

* Loss at 78° in vacuo over P₂O₅ for 60 hours. All other analytical values in this table are corrected for this loss.
† By the micro-Kjeldahl method.
‡ By turbidimetric analysis following Carius combustion (7).
§ By manometric CO₂ method (8).
∥ By colorimetric carbazole reaction (5).
¶ By a modification of the Elson and Morgan method (3) following hydrolysis in 4 N HCl for 14 hours at 100°.
** By chromic acid oxidation (9).
†† Theory for disaccharide repeating unit, C₁₄H₂₀O₁₁NNa.
‡‡ Theory for disaccharide repeating unit, C₁₀H₁₉O₁₁NSNa₂.

**TABLE III**

*Comparison of Action of Testicular and Streptococcal Hyaluronidase on Mucopolysaccharides from Different Sources*

<table>
<thead>
<tr>
<th>Mucopolysaccharide</th>
<th>Hyaluronidase preparation</th>
<th>Turbidity reduction*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Source</td>
<td>Enzyme units</td>
</tr>
<tr>
<td>Umbilical cord HA</td>
<td>Testicular</td>
<td>24</td>
</tr>
<tr>
<td>Rabbit skin HA</td>
<td>Streptococcal</td>
<td>24</td>
</tr>
<tr>
<td>Cartilage CSA</td>
<td>Testicular</td>
<td>24</td>
</tr>
<tr>
<td>Rabbit skin CSA</td>
<td>Streptococcal</td>
<td>24</td>
</tr>
<tr>
<td>Beef lung β-heparin</td>
<td>Testicular</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Streptococcal</td>
<td>2400</td>
</tr>
<tr>
<td>Pig gastric mucosa polysaccharide B‡</td>
<td>Testicular</td>
<td>1000</td>
</tr>
<tr>
<td></td>
<td>D‡</td>
<td>24</td>
</tr>
</tbody>
</table>

* Turbidimetric assays were performed as described previously (16, 17). Turbidity reduction is assumed proportional to the extent of depolymerization by enzyme.
† The β-heparin was a gift from Dr. A. Winterstein of F. Hoffmann-La Roche and Company, Basel, Switzerland.
‡ The polysaccharides B and D from pig gastric mucosa were gifts from Dr. H. Smith, Experimental Station, Porton, England.
crystals from HA agreed with an authentic sample of glucosamine hydrochloride, while that of the crystalline hexosamine from the CSA agreed with an authentic sample of chondrosamine hydrochloride from the CSA of bovine cartilage. Both patterns agreed with those published by Blix et al. (15) for the hydrochloride of glucosamine and chondrosamine.

Effect of Hyaluronidase—The HA isolated from rabbit skin was found to be completely digested by both testicular and streptococcal hyaluronidase (Table III). The CSA fraction, however, was resistant to the action of the hyaluronidase from streptococci, but was attacked some 30 per cent by the testicular enzyme. Table III includes also, for comparison, the effect of testicular hyaluronidase on acid polysaccharides with chemical analyses similar to those of the CSA from skin and cartilage. It will be noted that the β-heparin of Marbet and Winterstein (18) and the polysaccharide B isolated from pig gastric mucosa by Smith and Gallop (19) are not affected by the testicular enzyme, while the enzyme acts similarly on polysaccharide D (19) from pig gastric mucosa and on cartilage CSA.

DISCUSSION

The need for an adequate method for the isolation of the mucopolysaccharides from the ground substance of connective tissue has long been recognized. Gardell et al. (2) demonstrated two components in an extract of the mucopolysaccharides of pig skin, both moving toward the anode when subjected to slab electrophoresis in 0.1 M acetate buffer, pH 4.7. No chemical analyses of the fractions were undertaken. The same technique was applied subsequently by Bostrom and Gardell (4) to the separation of the acid polysaccharides from rat skin. In their report chemical analyses of the two components were limited to those of sulfur and hexosamine, both of which were considerably below theory for CSA.

With the HA fraction displaced toward the cathode in the system as described in the present communication, the two mucopolysaccharide fractions migrated in opposite directions. "Tailing" by the faster moving component did not contaminate the slower component. Complete chemical analyses of the skin HA and CSA fractions demonstrated a high degree of purity.

No claim is made for a quantitative recovery of the mucopolysaccharides from the skin since the purpose of the investigation was the isolation of analytically pure compounds rather than the exact measurement of the amount present. Furthermore, no claim is made that the acid polysaccharides were isolated in their highly polymerized native state.

The HA isolated from rabbit skin behaved in all respects like that from umbilical cord. The CSA from skin differed, however, from that of cartilage. This difference has been observed by Meyer and his collaborators.
(1, 12). That the CSA from rabbit skin is attacked some 30 per cent by testicular hyaluronidase is at variance, however, with the publication of Meyer and Rapport (12), who reported that the CSA isolated from pig skin is resistant to testicular hyaluronidase.

The low carbazole values for uranic acid obtained with the rabbit skin CSA fraction are of interest. Others have reported a similar finding for sulfated polysaccharides, notably β-heparin from beef lung (18) and polysaccharide B from pig gastric mucosa (19), both of which appear to be chemically similar to the CSA from skin and cartilage.

The possibility that two different sulfated mucopolysaccharides with the same chemical composition are present in skin must be considered. The isolation of two polysaccharides, analytically similar but enzymatically distinguishable, from beef lung (16) and from pig gastric mucosa (17), lends support to such a possibility and requires further investigation.

SUMMARY

A method is described for the isolation of hyaluronic acid (HA) and a chondroitinsulfuric acid (CSA) fraction in a high state of purity from the skin of rabbits.

The HA from skin appeared to be identical with that from umbilical cord. The CSA from skin differed from that of cartilage in its specific rotation, color reaction with carbazole for uronic acid, and as a substrate for testicular hyaluronidase. It is postulated that the CSA fraction from skin is a mixture of two sulfated polysaccharides.

BIBLIOGRAPHY

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