Isolation of Hyaluronic Acid from Broth Cultures of Streptococci*

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(Received for publication, September 12, 1963)

A readily available source of pure hyaluronic acid is a necessity in studies involving the ground substance of connective tissue and its relation to diverse spreading factors thought to be important in the pathogenesis of many bacterial diseases.

Any objection that contamination with other mucopolysaccharides or protein of hyaluronic acid prepared from mammalian sources is generally likely, unless additional purification and separation procedures are resorted to, can be overcome by using certain bacterial species as a source of pure hyaluronic acid.

Isolation of hyaluronic acid from group A hemolytic streptococci was first reported by Kendall, Heidelberger, and Dawson (1), who used acetate and ethanolic precipitation on their culture supernatants; between 60 and 140 mg of hyaluronic acid per liter of culture were obtained. Similar methods and results were reported by Seastone (2) and Topper and Lipton (3) with group A streptococcal strains in broth media.

Rosenman, Moses, Ludowieg, and Dorfman (4) obtained yields of purified hyaluronic acid from fluid cultures of group A streptococci, strain A111, of from 200 to 300 mg from 4 liters of medium with an acetone-phosphomolybdic acid-acetone precipitation technique.

Working with the same strain of microorganism, Cifonelli and Mayeda (5) described a simplified method of obtaining purified hyaluronic acid from streptococci and other biological sources by passing ethanol-precipitated crude mucopolysaccharide repeatedly through Darco G-60 cellulose pads. They obtained 300 to 400 mg of hyaluronic acid per liter of culture medium.

Warren and Gray (6) developed a semisynthetic medium for the cultivation of group A streptococci in large batches. Their methods for rapid, reproducible isolation of partially purified hyaluronic acid from the fluid media filtrates of group A streptococci consisted of acetone, acetate, and ethanolic precipitation with yields of 250 mg per liter of culture medium.

Our experiments concern the isolation and purification of hyaluronic acid from strains of group A hemolytic streptococci with 85% (NH₄)₂SO₄ concentration of the broth cultures for precipitation of mucopolysaccharide as suggested by Stock and Lynn (7). The method is relatively easy, reproducible, and gives a satisfactory amount of a highly purified hyaluronic acid.

**Experimental Procedure**

Some of the strains of group A hemolytic streptococci used in this study were strain 32369 and strain Coburn R18 from the New York State Laboratories, Department of Health, Albany, New York, and Cook B strain (Stock and Lynn (7)).

All strains were grown for 24 hours in mass culture, dialyzed Todd-Hewitt broth with added glucose and sodium bicarbonate (Stock and Lynn (7)) and were supplied to our laboratories through the courtesy of Dr. A. H. Stock. Most proteins (particularly the β toxins) were precipitated by the addition of 500 g of ammonium sulfate per liter of broth culture fluid and removed by filtration through Buchner funnels. Formalin, 2%, was added to the filtrate to prevent any further bacterial growth.

Then, 150 g of (NH₄)₂SO₄ per liter of solution were added slowly, with stirring, and the whole was allowed to stand at 4° for 72 hours. The final concentration of (NH₄)₂SO₄ for mucopolysaccharide precipitation was therefore 65%.

The precipitate was then filtered off under vacuum through Whatman No. 50 paper in Buchner funnels, the filter paper was cut carefully into small pieces, and the precipitate was washed off the paper with a minimal amount of distilled water. Higher yields have been obtained by collecting the precipitate in the Szent-Györgyi-Blum continuous flow attachment to the Servall centrifuge at 0° and 16,000 r.p.m. The solution containing the mucopolysaccharide was dialyzed at 4° against distilled water until no precipitate was discernible with BaCl₂. Initial stirring and the upper layer containing the mucopolysaccharide was dialyzed at 4° against distilled water until no precipitate was discernible with BaCl₂. Initial stirring and discarding of the chloroform layer (Sevag (8)) was followed by two or three further extractions until only a very faint ring of precipitate appeared at the chloroform interface. The chloroform-butanol layer was then discarded from a separate funnel, and the upper layer containing the mucopolysaccharide was poured into 1.5 volumes of absolute ethanol containing 5% sodium acetate and 2.5% glacial acetic acid (with a resultant pH of 4.5) and stored at 4° for 48 hours.

The resultant precipitate was then centrifuged (5 minutes at 3000 r.p.m.), the ethanol decanted, and the mucopolysaccharide washed with more absolute ethanol and then dissolved in a minimal volume of water. Reprecipitation with 5 volumes of ethanol, to which had been added a few drops of half-saturated sodium acetate solution, was then performed overnight at 4°.

The final precipitate was recovered by centrifugation (30 minutes at 5000 r.p.m.), washed in 100% ethanol, redissolved in water, and lyophilized to dryness. Although the results depended on the strain of microorganism originally used, all yields were very high, ranging from 0.5 to 1.0 g per liter of original broth culture.

**Results**

The bacterial mucopolysaccharide precipitate was then subjected to chemical analysis, the results of which are partially...
summarized in Table I. Nitrogen determinations, with the micro-Kjeldahl technique and the Aminco digestion apparatus, at first gave values averaging between 2.6% and 2.7% N₂. However, with exhaustively dried samples, the values consistently averaged 3.3% N₂; this supports the report by Roseman, Moses, Ludowieg, and Dorfman (4) that hyaluronic acid rapidly absorbs water during weighing, thus contributing to false nitrogen values. No protein could be detected by the Folin-Ciocalteu procedure (Kabat and Mayer (9)). Uronic acid was assayed by the method of Dische (10) with the use of the carbazole reaction. The acid hydrolysis method outlined by Boas (11) gave values averaging 44.9% uronic acid, whereas the resin hydrolysis method used by Meserschmid and Common (12) gave uronic acid values of 47.7%.

Hexosamine determinations were done according to the modification of the Elson-Morgan technique suggested by Boas (11). Values averaged 44.9% hexosamine. Nucleic acid contamination as detectable by phosphate determination (Hares and Isherwood (13)) was negative. Optical rotation was \([\alpha]_D^{24} = -70^\circ (c, 0.1 \text{ in water})\).

Viscosity measurements (Ostwald-Cannon-Fenske viscometers) relative to distilled water were run at 24° on freshly prepared samples (1 mg per ml concentration). The \(\eta_{av} H_2O, 24^\circ\), of 3.9 agrees reasonably well with figures presented by Rogers (14), and Cifonelli and Mayeda (5).

Degradation of the bacterial mucopolysaccharide was performed chemically with 0.5 m H₂SO₄ at 100° for varying time periods and with mild HCl hydrolysis in the presence of Dowex 50 resin for 16 hours as recommended by Anastassiadis and Common (12). After the necessary neutralization and separation procedures were performed, paper chromatographic analysis of the samples was conducted on Whatman No. 1 paper with the use of an ascending front of water-saturated collidine solvent and the spots then detected by the Elson-Morgan reaction (Partridge (15)) and aniline-hydrogen phthalate (Partridge (16)). Results showed only the presence of one uronic acid spot with a mobility corresponding to glucuronic acid, and one hexosamine spot corresponding in mobility to glucosamine.

Further support for the chemical nature of the bacterial product was provided by the preparation of hyaluronic acid from the material which was homogeneous chromatographically and with \(R_P\) and nitrogen values that corresponded with those reported by Weissmann and Meyer (17).

Enzymatic degradation of the purified mucopolysaccharide with commercial testicular hyaluronidase and subsequent paper chromatographic analysis yielded results roughly similar to those reported by Linker, Meyer, and Weissman (18), Linker, Meyer, and Hoffman (19), and Dodgson (20) and are indicative of hyaluronic acid.

Column chromatography with Dowex AG1-X2 anion exchange resin (California Corporation for Biochemical Research) according to the methods of Schiller, Slover, and Dorfman (21) and on Ecteola cellulose as reported by Ringertz and Reichard (22) was performed on aliquots of the mucopolysaccharide extract and 11-mL fractions collected on a Research Specialties Company fraction collector. Stepwise elution with 0.1, 0.5, 1.25, 1.5, and 2.0 m NaCl solutions successively applied was effected in 30 tubes per NaCl concentration. Determination of the recovery was estimated by the carbazole reaction (Dische (10)).

The streptococcal mucopolysaccharide was eluted almost entirely in the third and fourth tubes with 0.5 m NaCl from the Dowex columns and in the first three tubes with 0.1 m NaCl from Ecteola columns. No peaks occurred in the other fractions; recovery was 102%.

**DISCUSSION AND CONCLUSIONS**

Although mucopolysaccharides are to be found in varying proportions in all connective tissue, the highest quantitative yields are reported from human umbilical cord (Meyer and Palmer (23), Hadidian and Firie (24), and Jeanloz and Forchielli (25)), bovine vitreous humor (Meyer and Palmer (26)), and cock's comb (Boas (27)). In all cases, the need arises to separate the hyaluronic acid from the mucopolysaccharides or proteins present in the tissue (Caputo and Marcano (28) and Hallen (29)) and many methods have been described to effect the necessary further purification.

Cifonelli and Mayeda (5) passed umbilical cord and vitreous humor extracts over a Darco cellulose pad, whereas Pigman, Rizvi, and Holley (30) employed the electro-deposition method of Roseman, Watson, Duff, and Robinson (31) to separate hyaluronic acid from bovine synovial fluid. More recently, Matsumura, DeSalesgui, Herp, and Pigman (32) separated hyaluronic acid with 5% cetylpyridinium chloride solution from this same material. In other experiments with cock's wattles and rabbit skin, we have employed a modified method of proteolytic enzyme and mild alkali hydrolysis proposed by Schiller, Slover, and Dorfman (21), who have also used cetylpyridinium chloride to free the mucopolysaccharides of protein. For similar purposes, Berman (33) has described the use of DEAE-Sephadex (A-50, medium).

The experiments reported herein circumvent the need for the excess treatment involved in purification procedures of hyaluronic acid.

**Table I**

<table>
<thead>
<tr>
<th>Analysis Method</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micro-Kjeldahl</td>
<td>2.3%</td>
</tr>
<tr>
<td>Folin reaction</td>
<td>Negative</td>
</tr>
<tr>
<td>Elson-Morgan (Boas (11))</td>
<td>44.0%</td>
</tr>
<tr>
<td>Carbazole (Dische (10))</td>
<td>47.3%</td>
</tr>
<tr>
<td>Dowex, chromography</td>
<td>-70°</td>
</tr>
<tr>
<td>Phosphate (Hares and Isherwood (13))</td>
<td>3.90</td>
</tr>
<tr>
<td>Paper chromatography</td>
<td>Negative</td>
</tr>
<tr>
<td>Dowex 1, Ecteola, chromatography</td>
<td>Oligosaccharides</td>
</tr>
<tr>
<td>Glucuronic acid, glucosamine</td>
<td>Glucuronic acid, glucosamine</td>
</tr>
</tbody>
</table>
acid from mammalian sources. The results indicate, without much equivocation, that the material obtained from the group A hemolytic streptococcal broth culture is hyaluronic acid in relatively pure form. Although no sulfate or bacterial hyaluronidase activity determinations have been performed on the material, the chromatographic and electrophoretic analyses and the viscosity and optical rotation values rule out the possibility of any significant contamination with other mucopolysaccharides. The presence of protein seems to be ruled out by the nitrogen values and negative Folin reaction.

Besides the relative ease of preparation, the method described provides a readily obtainable source of hyaluronic acid in reasonable yield.

SUMMARY

A method is described whereby hyaluronic acid of relatively pure nature is readily obtainable in good yields from fluid cultures of group A hemolytic streptococci. The procedure involves increasing the ammonium sulfate concentration ion to 65% after removal of protein at the 50% concentration level. The results of the chemical analysis of the mucopolysaccharide so obtained are presented and indicate the presence of hyaluronic acid solely.

REFERENCES
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