PREPARATION AND SOME PROPERTIES OF HYALURONIC ACID FROM UMBILICAL CORD OF THE PIG*

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Within the last 13 years hyaluronic acid has been isolated from a variety of sources (1): umbilical cord, vitreous humor, synovial fluid, tumors, and hemolytic streptococci. These mucopolysaccharides all have essentially the same chemical composition, equimolecular portions of acetylglucosamine and glucuronic acid, but vary greatly in viscosity. Hyaluronic acid was first isolated by Meyer and Palmer (2) in 1934 from bovine vitreous humor. Human umbilical cord was shown by them (3) to be a rich source of hyaluronic acid. Recently Hadidian and Pirie (4) have prepared from this same source a product more viscous than any hitherto obtained. However, the umbilical cord of no species other than the human has yet been investigated as a source of hyaluronic acid. It was the purpose of this work to investigate pig umbilical cord as a source and examine the properties of the hyaluronic acid derived.

EXPERIMENTAL

Fractionation—Fresh frozen pig umbilical cords were used as the starting material in this work. In previous work, human umbilical cords were stored in acetone 1 to 6 weeks before processing. In this work, the effect of this acetone storage on the extraction procedures was studied by extracting a portion of fresh frozen pig umbilical cords as received, while a second portion was set aside in acetone for 6 weeks before the extraction of hyaluronic acid. The method of extracting hyaluronic acid was essentially the fractionation technique described by Hadidian and Pirie (4). The umbilical cords were cut up and extracted with water and the residue was ground and extracted with 0.1 M sodium chloride. At this point in the procedure an innovation was introduced; the residue from the sodium chloride treatment was extracted with boiling water. The residue was suspended in 0.01 M hydrochloric acid (the pH readjusted to 2 when necessary) and

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digested at 37° with pepsin, followed by trypsin at pH 7.6. Hyaluronic acid was precipitated from the respective fluids in the following manner: Hydrochloric acid was added and formed a “mucin clot” which was removed; 1½ volumes of ethanol were added to the acid fluid and the precipitate removed; solid ammonium sulfate was added to the alcoholic supernatant fluid with vigorous mechanical agitation, and on standing the system separated into two layers with hyaluronic acid appearing at the interface. A precipitate was not always obtained at each step in the procedure.

In this manner, both the fresh frozen pig umbilical cords and the acetone-stored cords were extracted. For purposes of comparison, human umbilical cords (acetone-stored) were extracted according to the above procedure. Supplementary work was done with pig cords by grinding fresh frozen material, and the entire mass incubated with pepsin at 37°, followed by trypsin. This digested fluid was put through the acid-alcohol-ammonium sulfate fractionation.

Purification—The mucin clots were partially fractionated by precipitation with alcoholic potassium acetate from alkaline solution, as in McClean’s (5) procedure, or incubation with pepsin and trypsin, the digestion procedure being more satisfactory usually.

Several of the preparations referred to in Table I were made by removing the protein by shaking with a mixture of 1 part chloroform and 2 parts amyl alcohol (volume per volume) according to the Sevag technique (6). The hyaluronic acid was precipitated from acid solution by 1½ volumes of ethanol, then suspended in a smaller volume of water and dialyzed.

Chemical Analyses—The nitrogen content was determined by the micro-Kjeldahl procedure. The acetyl content was measured by hydrolyzing a 1 ml. sample in 2.5 N H₂SO₄ at 100° for 75 minutes, steam-distilling in the apparatus described by Markham (7), and titrating the distillate with 1/75 N NaOH. The glucosamine determinations were made by the method of Elson and Morgan (8) on material that had been hydrolyzed for 6 to 8 hours in 5 N HCl at 100° and then evaporated to dryness in a vacuum desiccator over a moistened caustic. All of these analyses were made on aliquots of dialyzed solutions whose concentration had been determined by drying at 100° for 2 hours and cooled in a desiccator. The values refer to the free acid and not to a salt.

Viscosity Measurements—Viscosity measurements were made in an Ostwald viscosimeter with a capillary 9 cm. long and having a flow time of about 30 seconds for 4 ml. of distilled water. This volume was used in all the experiments reported, and all the measurements were made at 25°. The standard salt concentrations used throughout were 0.05 M NaCl and 0.05 M phosphate buffer at pH 7.0. This concentration of salt and buffer
### Table I

**Analyses of Mucopolysaccharides Isolated from Umbilical Cord**

<table>
<thead>
<tr>
<th>Extraction</th>
<th>Sample No.</th>
<th>Treatment</th>
<th>Nitrogen per cent</th>
<th>Glucosamine per cent</th>
<th>Acetyl per cent</th>
<th>Relative viscosity</th>
<th>Half time sec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 m NaCl</td>
<td>1</td>
<td>Interfacial</td>
<td>12.1</td>
<td></td>
<td></td>
<td>1.01</td>
<td></td>
</tr>
<tr>
<td>Hot water</td>
<td>2</td>
<td>&quot;</td>
<td>5.8</td>
<td></td>
<td></td>
<td>1.02</td>
<td></td>
</tr>
<tr>
<td>Pepsin-trypsin</td>
<td>3</td>
<td>Portion, 1.33 vols. ethanol</td>
<td>5.5</td>
<td>20.0</td>
<td>6.6</td>
<td>1.04</td>
<td></td>
</tr>
<tr>
<td>digest</td>
<td>4</td>
<td>&quot;</td>
<td>5.3</td>
<td></td>
<td>7.9</td>
<td>1.06</td>
<td></td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>5</td>
<td>Samples 3 and 4 combined, Sevag purification</td>
<td>3.5</td>
<td>31.4</td>
<td>7.7</td>
<td>1.08</td>
<td></td>
</tr>
<tr>
<td>Mucin clots</td>
<td>6</td>
<td>Alcoholic potassium acetate purification</td>
<td>3.7</td>
<td>23.4</td>
<td>6.6</td>
<td>1.03</td>
<td></td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>7</td>
<td>Sample 6, Sevag purification</td>
<td>3.1</td>
<td>34.6</td>
<td>7.9</td>
<td>1.03</td>
<td></td>
</tr>
</tbody>
</table>

Yield: gross, 13.2%; corrected to 3.6% N, 5.4%

### B. Pig (fresh frozen; acetone-stored)

<table>
<thead>
<tr>
<th>Extraction</th>
<th>Sample No.</th>
<th>Treatment</th>
<th>Nitrogen per cent</th>
<th>Glucosamine per cent</th>
<th>Acetyl per cent</th>
<th>Relative viscosity</th>
<th>Half time sec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 m NaCl</td>
<td>10</td>
<td>Mucin clot, alcoholic CH₃COOK purification</td>
<td>6.5</td>
<td></td>
<td></td>
<td>1.03</td>
<td></td>
</tr>
<tr>
<td>0.1 &quot; &quot;</td>
<td>11</td>
<td>1.33 vols. ethanol</td>
<td>5.2</td>
<td>31.5</td>
<td>8.1</td>
<td>1.31</td>
<td>760</td>
</tr>
<tr>
<td>0.1 &quot; &quot;</td>
<td>12</td>
<td>Sample 11, Sevag purification</td>
<td>3.2</td>
<td>37.9</td>
<td>10.2</td>
<td>1.38</td>
<td>482</td>
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<tr>
<td>0.1 &quot; &quot;</td>
<td>13</td>
<td>Interfacial</td>
<td>9.5</td>
<td></td>
<td></td>
<td>1.03</td>
<td></td>
</tr>
<tr>
<td>Hot water</td>
<td>14</td>
<td>&quot;</td>
<td>3.5</td>
<td>34.9</td>
<td>8.5</td>
<td>1.15</td>
<td>920</td>
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<tr>
<td>Pepsin-trypsin</td>
<td>15</td>
<td>Mucin clot</td>
<td>10.6</td>
<td></td>
<td></td>
<td>1.01</td>
<td></td>
</tr>
<tr>
<td>digest</td>
<td>16</td>
<td>1.33 vols. ethanol</td>
<td>5.4</td>
<td>34.1</td>
<td>6.5</td>
<td>1.07</td>
<td></td>
</tr>
<tr>
<td>&quot; &quot;</td>
<td>17</td>
<td>Sample 10, Sevag purification</td>
<td>3.2</td>
<td>31.3</td>
<td>9.0</td>
<td>1.08</td>
<td></td>
</tr>
</tbody>
</table>

Yield: gross, 5.3%; corrected to 3.6% N, 3.3%

### C. Pig (fresh frozen)

<table>
<thead>
<tr>
<th>Extraction</th>
<th>Sample No.</th>
<th>Treatment</th>
<th>Nitrogen per cent</th>
<th>Glucosamine per cent</th>
<th>Acetyl per cent</th>
<th>Relative viscosity</th>
<th>Half time sec.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pepsin-trypsin</td>
<td>20</td>
<td>1.33 vols. ethanol</td>
<td>5.6</td>
<td>30.6</td>
<td>8.1</td>
<td>1.17</td>
<td></td>
</tr>
<tr>
<td>digest</td>
<td>21</td>
<td>Sample 20, Sevag purification</td>
<td>3.6</td>
<td>29.0</td>
<td>7.8</td>
<td>1.15</td>
<td>957</td>
</tr>
</tbody>
</table>

Yield: gross, 10.0%; corrected to 3.6% N, 6.6%
was used, since it was the one found by Hadidian and Pirie (9) to give optimum results. The term “relative viscosity” refers to the ratio of flow time of the test solution to the flow time of a solution with the same salt concentration. For measuring the relative viscosity of hyaluronic acid preparations a standard concentration of 0.3 gm. per liter was adopted.

The rate at which hyaluronic acid preparations lost viscosity with bull testis hyaluronidase was measured by incubation at 25° with the enzyme in the standard ionic environment. The time required for the viscosity to fall half way from the initial to the presumed final value was taken as a measure of the rate of enzyme action. Half time measurements are customary in studies of this enzyme. Partially purified bull testis enzyme (Schering) was used in all of these experiments at a concentration of 50 γ in 4 ml. of test solution.

**Preparation of Inhibitors**

_Nitration_—A few hyaluronic acid preparations were nitrated to test their inhibition of hyaluronidase hydrolysis of hyaluronic acid. The nitration
procedure used was that described by Hadidian and Pirie (9). Both the acid-insoluble and the acid-soluble portions were tested for their inhibitory effect at a concentration of 0.03 gm. per liter. The nitrogen content was determined by the micro-Kjeldahl method modified to include the nitrogen of nitrates.

Acetylation—Preparations from pig cord were acetylated to study their inhibitory effect on the hyaluronidase-hyaluronic acid system. The acetylation procedures used were the two described by Hadidian and Pirie (9). In the sulfuric acid method acetylation was allowed to proceed for 30 to 45 minutes at 27°. Acetylation was continued for 2 hours at 20° with the pyridine method. The acetyl content was determined by the method used above for hyaluronic acid preparations and included both O-acetyl and N-acetyl.

Results

Umbilical Cord—Pig umbilical cord proved to be a rich source of hyaluronic acid, nearly as rich as human cord. The yield of hyaluronic acid obtained from acetone-stored material was 3.3 per cent of the dry weight of the pig cord as compared with 4.4 per cent for human cord (these values corrected to 3.6 per cent nitrogen).

The most striking difference observed (Table I) for the series of pig cord preparations was in the relative viscosity; all fresh pig cord preparations were very low in viscosity, whereas one product in particular from defatted pig cord (Sample 12) with a relative viscosity of 2.3, calculated to a concentration of 1 gm. per liter, compared very favorably with the better preparations from various sources reported by other investigators. A compilation of these has been made by Hadidian and Pirie (4). The values for the chemical constituents of preparations from fresh frozen cords were lower than those from defatted cords. It was also noted that for pig cord in general the viscous preparations contained 8.5 to 10.2 per cent acetyl, but preparations made by enzymic digestion of residues had lower acetyl contents, as low as 6.5 per cent. There was a comparable deficiency in the glucosamine content. Viscous products contained 35 to 38 per cent, whereas the non-viscous had less than 35 per cent. The nitrogen values were in the range of 3.1 to 3.5 per cent.

The fresh frozen pig cords which were ground and subjected to pepsin-trypsin digestion without prior treatment produced the highest yield of hyaluronic acid. Since no fractionation was involved in this procedure, the relative viscosity and acetyl and glucosamine values were midway between those for the viscous and non-viscous preparations obtained previously. This was, of necessity, anticipated, as this preparation represented the average of the heterogeneous hyaluronic acid fractions present. As has
Hyaluronic Acid in Umbilical Cord

been observed in previous work precipitating hyaluronic acid, the pepsin-
trypsin digestion solution yielded no mucin clot with concentrated HCl and
no interfacial material separated, but the precipitate was obtained with
alcohol. This crude material was not excessively contaminated with
protein; the nitrogen content was 5.6 per cent which dropped to 3.6 per cent
on purification by the Sevag technique followed by dialysis. The half time
of 957 seconds for Sample 21 was about what would be expected from a
preparation having a relative viscosity of 1.15.

Inhibitors—Three pig cord products, Samples 5, 12, 21, were nitrated;
both the acid-insoluble and the acid-soluble fractions were tested for their
inhibiting effect on the hyaluronidase-hyaluronic acid system. Sample 5
was non-viscous, Sample 12 the most viscous pig product, and Sample 21

<table>
<thead>
<tr>
<th>Description of Inhibitor</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 5. Nitrated, acid-insoluble</td>
<td>2.1</td>
</tr>
<tr>
<td>&quot; 12. &quot;</td>
<td>1.6</td>
</tr>
<tr>
<td>Samples 5, 12. Nitrated separately, acid-soluble fractions combined</td>
<td>4.8</td>
</tr>
<tr>
<td>Sample 21. Nitrated, acid-insoluble, 5.9% N</td>
<td>1.5</td>
</tr>
<tr>
<td>&quot; 21. &quot; acid-soluble, 4.7% N</td>
<td>2.1</td>
</tr>
<tr>
<td>&quot; 21. Acetylated, sulfuric acid method, 18.2% acetyl</td>
<td>1.3</td>
</tr>
<tr>
<td>&quot; 21. &quot; pyridine method, 18.4% acetyl</td>
<td>1.2</td>
</tr>
</tbody>
</table>

intermediate. The acid-soluble fractions of Samples 5 and 12 were com-
bined before measuring their inhibition. This was done because of the
similarity in the inhibition given by their acid-insoluble products. The
values obtained for all of the inhibitors with a brief description of the
derivation of each are given in Table II. The acid-soluble products were
found to be better inhibitors than the acid-insoluble fractions. Of these
nitrated products, the one derived from the non-viscous preparation gave
the greatest inhibition. Nitrogen analyses (modified micro-Kjeldahl)
made on the nitrated products from Sample 21 indicated that the acid-
insoluble material was nitrated to the extent of one —NO₂ group, and the
acid-soluble one half an —NO₂ group. The inhibition found for these
nitrated pig cord preparations was of about the same order of magnitude as
that reported by Hadidian and Pirie (9) for their inhibitors made from
human cord products.
One hyaluronic acid preparation from pig cord was acetylated by both the sulfuric acid catalyst method and the pyridine method; by each method the resulting product contained about 18 per cent acetyl. Acetylated products were found to be poorer inhibitors than the nitrated acid-soluble products.

**DISCUSSION**

Hyaluronic acid can be extracted from pig cord and purified by the same methods that have been used for human umbilical cord. The products prepared from pig cord contained practically the same proportion of nitrogen, acetyl, and glucosamine as preparations from human cord. The general trend in the physical and chemical properties noted by Hadidian and Pirie (4) for human cord preparations was found here to be the same for pig cord; i.e., mucin clots had the lowest viscosity with material from pepsin-trypsin-digested extracts intermediate between those and the viscous products, and the non-viscous preparations contained less than the theoretical percentage of both acetyl and glucosamine. Preparations of hyaluronic acid from pig cord were hydrolyzed by the enzyme hyaluronidase (testis). Nitrated and acetylated pig preparations were found to inhibit the hyaluronidase-hyaluronic acid system to the same extent as inhibitors made from human cord.

In comparing the physical and chemical properties of hyaluronic acid preparations from pig umbilical cord with those from human cord, the most obvious difference was found in the relative viscosity; the human cord products were appreciably more viscous. However, the half time of products from both sources was found to be inversely proportional to the relative viscosity. The acetyl and glucosamine content of human preparations ranged, in general, higher than in pig preparations. These differences in properties observed may be attributable to a species difference.

The quality of pig cord preparations extracted was enhanced by acetone storage prior to processing for hyaluronic acid. Apparently a protein denaturation process was involved which decreased the solubility of the proteins since the crude products from the fresh frozen pig cords were contaminated with protein to a far greater extent than those from the defatted pig cords. The innovation of adding a boiling water treatment to the residue from the 0.1 M NaCl extraction in the procedure was of no particular efficacy. A substantial amount of protein was extracted by boiling water along with a small amount of hyaluronic acid.

**SUMMARY**

Pig umbilical cord was found to be nearly as rich a source of hyaluronic acid as human umbilical cord. The products derived from pig cord contained approximately the same proportion of nitrogen, acetyl, and glucosamine as human cord products and were also hydrolyzed by the enzyme
hyaluronidase. Hyaluronic acid preparations from pig cord were nitrated and acetylated in the same manner, and inhibited the hyaluronidase-hyaluronic acid system to about the same extent as similar inhibitors made from human cord preparations. In comparing the hyaluronic acid preparations from pig umbilical cord with those from human cord, the essential difference was in the relative viscosity. The pig cord products were appreciably less viscous.

Acetone storage of pig cords prior to processing enhanced the quality of the hyaluronic acid obtained and simplified the extraction and purification procedures, although the introduction of a boiling water treatment into the extraction procedure offered no particular advantage.

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