Studies in Histochemistry

II. MICRODETERMINATION OF HYALURONIDASE AND ITS INHIBITION BY FRACTIONS OF ISOLATED MAST CELLS*

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(Received for publication, June 16, 1958)

There should be considerable use for a procedure for the measurement of hyaluronidase in histologically or cytologically defined samples, e.g. microtome sections of tissue, small groups of isolated cells, or even single large cells such as protozoa. Adaptation of turbidimetric methods to a microscale was undertaken for this reason, as well as for the immediate need of a method for the determination of hyaluronidase inhibition by fractions of isolated mast cells.

Two turbidimetric procedures were tested, one based on the method of Tolksdorf et al. (1), which is one of a number of modifications of turbidimetric methods reviewed by Tolksdorf (2), and the other based on the more recent method of Di Ferrante (3). The Tolksdorf method proved preferable for inhibitor studies, and it was adapted to micromeasurements requiring samples 0.01 to 0.001 of the usual magnitude.

The Di Ferrante method was found to be unsuitable for the present inhibitor study, because the high concentration of NaCl used (0.15 M) interfered with the enzyme inhibition by heparin. Thus, barely perceptible inhibition was given by 40 μg. of heparin per ml. of reaction mixture, although 5 μg. per ml. gave 80 per cent inhibition by the Tolksdorf method. Interference of the heparin inhibition by NaCl is well known; it has been discussed, for example, by Meyer (4) and by Alburn et al. (5). Another difficulty arises when magnesium ions are added to obtain optimal inhibition by the nonspecific inhibitor of blood. The turbidity which is developed by the alkaline reagent is influenced by the precipitation of magnesium hydroxide.

Mast cells contain heparin material, and heparin is a heat-stable inhibitor of hyaluronidase. The possibility that the heat-labile nonspecific hyaluronidase inhibitor in blood serum is derived from the heparin-protein complex of mast cells was raised by Glick and Ochs (6). Circumstantial evidence submitted by Glick and Sylven (7) seemed to be compatible with this possibility. Subsequently, Newman et al. (8) isolated from human blood plasma a carbohydrate-containing protein which was a hyaluronidase inhibitor with certain properties characteristic of the inhibitor of fresh plasma. The protein had 0.1 per cent sulfur as sulfate and therefore could not be a heparin-protein complex.

The possibilities remained that the protein described by Newman et al. is not the only heat-labile hyaluronidase inhibitor in serum or plasma and that mast cells contain not only heat-stable heparin material but also heat-labile hyaluronidase inhibitor which may be contributed to the blood. In this study a heat-labile inhibitor was not found in mast cells; in fact heating increased the inhibition by mast cell fractions. Reviews dealing with morphological and chemical constituents of mast cells have appeared recently (9-12).

EXPERIMENTAL AND RESULTS

Apparatus

The apparatus for the microadaptations was described in earlier studies in this series and in a recent review (13). Pyrex glass tubes, 35 mm. long and 5 mm. inside diameter, were used as reaction vessels. Constriction pipettes were used throughout. The optical measurements were made in a Beckman model DU spectrophotometer with Lowry-Bessey cuvettes, with or without Glick-Grunbaum inserts which use 6 μl. for a 1-cm. light path (14).

Micro Turbidimetric Procedure Adapted from Method of Tolksdorf et al.

Reagents—Bovine testicular hyaluronidase preparations were made by extraction at 3° of 0.5 gm. of fat-free dried testis powder with 100 ml. of 0.05 M phosphate buffer, pH 7, which was 0.05 M with respect to NaCl. The enzyme concentration was adjusted with the saline buffer to give a working solution which would produce a change in absorbance from between 0.6 and 0.7 to between 0.2 and 0.3 under the assay conditions which obtained. The Beckman instrument has maximal accuracy in this range (15). Human umbilical cord hyaluronate was also dissolved in the 0.05 M NaCl-phosphate buffer. The concentration was adjusted to give an initial assay turbidity equivalent to 0.6 to 0.7 absorbance. 0.05 M magnesium acetate was used so that the concentration in the final reaction mixture would be 0.01 M, the concentration found optimal to potentiate the nonspecific serum inhibitor under the conditions of the assay. 1 per cent bovine serum albumin (Fraction V, Armour and Company) in 0.5 M

* This investigation was supported by research grants, Nos. H2028 and RG 2911, from the National Institutes of Health, United States Public Health Service, and by a research grant from the Louis W. and Maud Hill Family Foundation.

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‡ Some of the data in this paper were included in a thesis submitted in partial fulfillment of the requirements of the Graduate School of the University of Minnesota for the degree of Master of Science.
**Microdetermination of Hyaluronidase**

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**Diagram 1**

300-μl suspension of disrupted mast cells in buffer*

<table>
<thead>
<tr>
<th>Supernatant granul suspension</th>
<th>Sediment, resuspended in 300 μl. buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 μl. used for microscopic examination</td>
<td>260 μl. centrifuged for 15 minutes at 3000 × g</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Supernatant</th>
<th>Granules, resuspended in 260 μl. of buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>150 μl. heated for 20 minutes at 65-70°, centrifuged for 15 minutes at 3000 × g</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fraction A</th>
<th>130 μl. heated for 20 minutes at 65-70°, centrifuged for 15 minutes at 3000 × g</th>
</tr>
</thead>
</table>

**Table I**

<table>
<thead>
<tr>
<th>Inhibitor preparation</th>
<th>Absorbance decrease</th>
<th>Inhibition</th>
<th>Inhibitor units per 10,000 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (without inhibitor)</td>
<td>0.343</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>Fraction A</td>
<td>0.340</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fraction B</td>
<td>0.343</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fraction C</td>
<td>0.208</td>
<td>39</td>
<td>1.5</td>
</tr>
<tr>
<td>Fraction C (diluted 2 times)</td>
<td>0.295</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Fraction D</td>
<td>0.022</td>
<td>85</td>
<td></td>
</tr>
<tr>
<td>Fraction D (diluted 9 times)</td>
<td>0.141</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>Fraction E</td>
<td>0.221</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Fraction F</td>
<td>0.335</td>
<td>2</td>
<td>0.1</td>
</tr>
<tr>
<td>Fraction F (diluted 2 times)</td>
<td>0.022</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>Fraction F (diluted 4 times)</td>
<td>0.181</td>
<td>47</td>
<td>7.9</td>
</tr>
<tr>
<td>Fraction G</td>
<td>0.203</td>
<td>7</td>
<td>0.7</td>
</tr>
<tr>
<td>Fraction H</td>
<td>0.118</td>
<td>65</td>
<td>2.8</td>
</tr>
<tr>
<td>Fraction H (diluted 2 times)</td>
<td>0.216</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

* Potassium phosphate buffer, 0.05 M, pH 7.0, in all steps.

**Fractionation of Mast Cells**

Mast cells were isolated from washings of peritoneal tissue from single rats according to the procedure described by Glick et al. (16). After centrifugation in the sucrose-Versene (the disodium salt of ethylenediaminetetraacetic acid)-phosphate density gradient, the cell layer was transferred to a small glass Potter-Elvehjem homogenizer tube and the cells were separated by centrifugation and washed with 1 ml. of Hanks' solution, again separated and washed with 1 ml. of 0.05 μ phosphate buffer, pH 7, and finally resedimented and taken up in 200 μl. of the phosphate buffer. Although this buffer was slightly hypotonic, no effect on the cells was visible microscopically. The cell suspensi-
sion was homogenized with an electrically driven pestle for 10 minutes, a period sufficient for disruption of practically all of the cells, and the pestle was rinsed with 100 µl of the buffer. The combined homogenate and rinsing solution was treated as shown in the flow diagram, which depicts mast cell fractionation (Diagram 1). Since the sediment from each centrifugation was resuspended in the volume from which it was centrifuged, equal volumes of each fraction were comparable with one another. Negligible amounts were removed from the original cell suspension for cell counting in a hemacytometer.

Hyaluronidase Inhibition by Mast Cell Fractions

To obtain a solution which would inhibit within the range linear to inhibitor concentration under the conditions of the experiment, certain fractions were diluted with the buffer 2 and 4 times. (Linearity was maintained up to 80 per cent inhibition.) Decrease in absorbance without inhibitor was 0.34 to 0.43 in different experiments. Data of a typical experiment are given in Table I. The initial sediment which contained many granules had considerable inhibitor (Fraction H), but the heating solubilized the inhibitor and increased it about 3 times (Fraction F). The initial supernatant which contained suspended granules as the only bodies visible by phase contrast microscopy exhibited no soluble inhibitor (Fractions A and B), but the granules had inhibitor (Fraction C) which could be solubilized and increased approximately 4 times by the heating (Fraction D). Thus it seems that the granules possess inhibitor, which is not heat-labile. Moreover, heating sufficient to destroy the heat-labile labile inhibitor in serum actually liberates or otherwise increases the effect of the inhibitor from mast cells.

SUMMARY

The procedure of Tolksdorf et al. for the turbidimetric determination of hyaluronidase was adapted to a microscale requiring 0.01 to 0.001 the usual amount of sample. The micromethod was applied to the measurement of hyaluronidase inhibition by fractions of mast cells isolated from peritoneal washings of the rat.

Inhibitor was found only in fractions containing granules. Inhibition by suspensions of granules was increased several-fold by heating at 65°-70° for 20 minutes, conditions which destroy the nonspecific inhibitor of blood serum. Thus it is unlikely that the latter is derived from mast cells.

Acknowledgments—The authors wish to thank Gunhild Otto-son and Dr. Tatiana Ivanov for technical assistance.

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