Microtest for Mucopolysaccharides by Means of Toluidine Blue

With Special Reference to Hyaluronic Acid

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Although a vast amount of literature has been published in recent years on the subject of hyaluronic acid and hyaluronidase, owing to the importance of a biologic function which has been ascribed to these two substances, no satisfactory method yet exists for the determination of minute quantities of hyaluronic acid or other mucopolysaccharides.

Various mucopolysaccharides form stable dispersions in water or buffer solutions. The stability of such suspensions can be destroyed by neutralizing the negative charges of the particles with charges of opposite sign. It has been possible in the present study to utilize the alkaline dye toluidine blue for this purpose. In the presence of the dye, hyaluronic acid and other mucopolysaccharides produce colored precipitates of characteristic morphology. Advantage has been taken of these properties to develop a microtest for the detection of minute quantities of hyaluronic acid and related mucopolysaccharides.

MATERIALS AND METHODS

1. Buffer solutions. pH 1.0-2.2: Clark & Lubs buffers, HCl-KCl (2); 
   pH 3.1-6.22: Michaelis buffers, acetic acid-sodium acetate (3); pH

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Fig. 1. Filamentous precipitate formed by the interaction of hyaluronic acid and toluidine blue (× 56). Fig. 2. Granular precipitate formed when the hyaluronic acid toluidine blue interaction product is stirred with a glass rod in the process of formation (× 150). Fig. 3. Precipitate formed by the interaction of toluidine blue with heparin solution, pH 2 (× 150). Fig. 4. Precipitate formed by the interaction of toluidine blue with chondroitin sulfuric acid, pH 2 (× 150).
Fig. 5. Precipitate formed by the interaction of toluidine blue with ox vitreous humor (× 56). Fig. 6. Precipitate formed by the interaction of toluidine blue with human normal vitreous humor (× 150). Fig. 7. Precipitate formed by the interaction of toluidine blue with myopic vitreous humor (× 150). Fig. 8. Precipitate formed by the interaction of toluidine blue with subretinal fluid (× 150).
7.8-10.0: Clark & Lubs buffers, boric acid, KCl-NaOH mixtures (2). Above pH 10.0, 0.01N NaOH was added to bring the solution to the desired pH.

2. Heparin (Hynson, Westcott & Dunning, Inc., Baltimore, Md.). Solutions containing 500 µg. per ml. were made up in the above series of buffers, and solutions ranging from 20 to 100 µg. per ml. were made up at pH 6.

3. Chondroitin sulfuric acid (Nutritional Biochemicals Corp., Cleveland, Ohio). Solutions containing 500 µg. per ml. were made up in the above series of buffers, and solutions ranging from 20 to 100 µg. per ml. were made up in buffer at pH 6.

4. Hyaluronic acid (human umbilical cord, Worthington Biochemical Co., Freehold, N. J.). Solutions containing 500 µg. per ml. were made up in the above series of buffers, and solutions containing 1.0, 10, 20, 30, 40, 50, and 100 µg. per ml. were made up in M/10 acetate buffer, pH 6.

5. Hyaluronidase ("Hyalase", Benger Laboratories Ltd., Cheshire, England). The contents of each 1000-unit ampule were dissolved in 1 ml. of M/10 acetate buffer, pH 6.

6. Dye (George T. Gurr, London). Toluidine blue (dimethyltoluidinechloride) was made up in 0.1 per cent solution in distilled water.

PROCEDURE

The reaction between mucopolysaccharide and dye is carried out by placing 1 drop of dye on a microscope slide and mixing with an equal quantity of buffered mucopolysaccharide test solution. A precipitate is obtained. The type of precipitate formed depends on the manner in which the 2 drops are mixed on the slide. Mixing by tilting the slide gently back and forth produces a precipitate consisting of elongated filaments and fibers (Fig. 1), but if the drops are mixed by stirring with a glass rod, which disrupts the fibers in the process of formation, a characteristic coarsely granular precipitate is obtained (Fig. 2). It is convenient to examine the slide for a precipitate about 10 minutes after the drops are mixed.

Precipitates of the same general type, but differing in form and color, were also obtained with heparin (Fig. 3), and chondroitin sulfuric acid (Fig. 4). Similarly, metachromatic precipitates were obtained with various optic fluids such as ox vitreous humor (Fig. 5), human normal and pathologic vitreous humor (Figs. 6 and 7), aque-
ous humor, subretinal fluid (Fig. 8), and extracts of lens and cornea. With fluids, the reaction was carried out by adding 1 drop of toluidine blue to 1 drop of test material diluted with one drop of buffer solution at pH 6. If a dry material such as conjunctival smears was tested, it was first dissolved in a minimum quantity of buffer, pH 6, and then mixed with an equal volume of toluidine blue on the slide. The addition of one drop of human serum to an equal quantity of toluidine blue caused the formation of a fine, powdery blue precipitate readily distinguishable from the filamentous or coarsely granular precipitate obtained in the reaction between hyaluronic acid and toluidine blue. Fibrin and toluidine blue produced a precipitate composed of blue globules distinctly different from the filamentous precipitates formed by the same dye with hyaluronic acid.

**EFFECT OF HYALURONIDASE**

Test tubes containing one drop of hyaluronic acid at a concentration of 200 µg. per ml. were incubated for various lengths of time at 37° with 50, 100, 200, and 1000 units of hyaluronidase. One drop of toluidine blue was added to each test tube. Increasing the concentration of hyaluronidase produces the same effect as prolonging the time of incubation—diminution of the length of the characteristic precipitate and finally no precipitation. Hyaluronic acid depolymerized to oligosaccharides as a result of enzymatic action produces no precipitate with toluidine blue. The formation of a precipitate between the various biologic fluids or extracts of the eye mentioned above and toluidine blue could similarly be precluded by prior incubation with hyaluronidase.

**SENSITIVITY OF METHOD**

The minimum quantity of hyaluronic acid detectable by the present method was shown by serial dilution to be 0.5 µg. (10 µg./ml.). In very dilute solutions of hyaluronic acid, a microscope is necessary to detect the precipitate.

Robertson, Ropes, and Bauer (4) have shown that hyaluronic acid in the presence of proteins precipitates in a fibrous clot when the solution is acidified with acetic acid. To compare the sensitivity of the present method with that of Robertson et al., protein-containing human vitreous humor was tested in various dilutions with the reagents employed in the two methods. The results are shown in Table 1. No precipitate could be detected by the modification of Robertson's
Table 1. Comparison of Hyaluronic-Acid-Detecting Methods

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>Dilution</th>
<th>Presence of precipitate</th>
<th>Macroscopic (Robertson)</th>
<th>Microscopic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>With acetic acid</td>
<td>With toluidine blue</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1:2</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>1:4</td>
<td>±</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>1:8</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>1:16</td>
<td>−</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>1:32</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>1:64</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

Test substance: human vitreous humor, serially diluted with buffer, pH 6. The reactions were carried out with one drop of the dilutions mentioned above.

The method under the microscope at dilutions of vitreous humor higher than 1:16, whereas the use of toluidine blue enabled the detection of positive-reacting material in vitreous humor in dilutions as high as 1:64.

EFFECT OF pH

The effect of pH on the reaction between mucopolysaccharides and dye was studied by mixing 1 drop of a solution of mucopolysaccharide in buffer solution at various pH's over the range from pH 1-14 with an equal quantity of toluidine blue dissolved in buffer at an identical pH. It was found that while all mucopolysaccharides tested have an optimum zone of precipitation between pH 6-10, heparin (a stronger acid) produces a precipitate at a pH as low as 1.4, whereas chondroitin sulfouric acid fails to give a precipitate at a pH lower than 2, and hyaluronic acid at a pH lower than 3.3. The characteristic colors of the precipitates at pH 6 are recorded in Table 2.

Table 2. Color of Precipitate Obtained on Mixing One Drop of Various Mucopolysaccharides (200 μg. per ml.) with One Drop of Toluidine Blue in Buffer Solution pH 6

<table>
<thead>
<tr>
<th>Mucopolysaccharide</th>
<th>Precipitate color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td>Purple</td>
</tr>
<tr>
<td>Chondroitin sulfuric acid</td>
<td>Purple-violet</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>Dark violet</td>
</tr>
</tbody>
</table>
DISCUSSION

The present investigation was undertaken when it was found to be impossible to detect hyaluronic acid in low concentrations in certain specimens of normal and pathologic eye fluids by any existing method. The quantitative method proposed by Tolksdorf (5), based on the amount of turbidity produced in hyaluronic acid solution at an acid pH by the addition of protein as read in a photoelectric colorimeter, can only be used when concentrations of hyaluronic acid greater than 32 μg. per ml. are present. Tests conducted on the sensitivity of the modified Robertson, Ropes, and Bauer method (4) showed that it was impossible to detect hyaluronic acid in concentrations lower than 30 μg./ml. in the presence of horse serum, or in vitreous humor in humor dilutions greater than 1:16, even when a microscope was used. The method presented in the present paper utilizing toluidine blue permits as little as 0.5 μg. of hyaluronic acid to be detected and affords a positive reaction with vitreous humor at a humor dilution of 1:64. By means of the present method and with pure hyaluronic acid at a concentration of 10 μg. per ml. (we use only 1 drop), it is possible to get positive results.

SUMMARY

A micromethod is presented for the detection of hyaluronic acid when as little as 0.5 μg. of material are present. The method is simple and applicable as well to some other mucopolysaccharides.

REFERENCES

2. Clark, W. M., Topics in Physical Chemistry, Baltimore, Williams & Wilkins, 1948.