A TURBIDIMETRIC METHOD FOR THE ASSAY OF HYALURONIDASE

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(Received for publication, September 26, 1947)

A variety of methods have been devised for the assay of the enzyme hyaluronidase. These may be divided into four types: (a) measurement of the "spreading effect" in the skin of rabbits and guinea pigs (1, 2), (b) reduction of viscosity (3, 4), (c) prevention of mucin clot formation (5), and (d) chemical measurement of total reducing substances, N-acetylglucosamine, or glucuronic acid liberated on the complete hydrolysis of hyaluronic acid (6, 7).

The first group of methods ("spreading activity") is most sensitive, but is time-consuming, requires large numbers of animals, and lacks specificity in that substances other than hyaluronidase have been shown to possess spreading activity. They are of comparatively little value for the routine assay of large numbers of preparations. The viscosity method has been used widely with considerable success. It is, however, tedious and time-consuming and requires large amounts of hyaluronic acid.

The original mucin clot prevention method is based on the fact that hyaluronic acid reacts with acidified albumin to form a clot (5). If hyaluronic acid is first acted on by hyaluronidase, such a clot does not form. This then affords a basis for the estimation of hyaluronidase.

It has been noted that when the smallest amount of enzyme necessary to prevent clot formation is used there remains a uniform turbidity. If larger amounts of enzyme are used, this turbidity does not develop.

The use of the various chemical methods is open to serious question in that Hahn (8) has recently shown that highly purified hyaluronidase does not bring about the complete hydrolysis of hyaluronic acid. The release of glucuronic acid and N-acetylglucosamine apparently depends upon the presence of contaminating enzymes in the crude preparations.

A method based on turbidity development seemed most likely to be rapid, simple, and accurate. Seastone (9) showed that under certain conditions turbidity is directly proportional to hyaluronic acid concentration. Kass and Seastone (10) used this fact in developing a quantitative turbidimetric method for the assay of hyaluronidase. They utilized acidified serum for turbidity development. Leonard, Perlman, and Kurzrok (11) have developed a similar method for the determination of hyaluronidase in semen by use of acidified serum.
It is the purpose of this communication to describe a modified turbidity method with crystalline horse serum albumin. The reagents used in this method are stable and easily reproducible. Exact conditions for the use of this method have been determined.

The turbidity method may be applied to the estimation of hyaluronidase in two ways. The first of these consists of the determination of the rate at which hyaluronic acid is hydrolyzed. This is analogous to the viscosity-reducing method. We have shown that this method is adequate and can be used in kinetic studies (12). It has the disadvantage of requiring a number of different determinations for each sample analyzed and is thus time-consuming. A simpler method consists of determining the amount of hyaluronic acid which remains after some specific time. This requires only one turbidity determination for each preparation being analyzed. The method described in this communication depends on such an estimation.

**Materials**

**Hyaluronic Acid**—Human umbilical cords were washed free of blood and stored under acetone in the cold. The acetone powder was prepared by grinding in a meat grinder and washing with acetone. 200 gm. of dry powder, 1200 ml. of Hayem’s solution, and 3000 ml. of water were stirred vigorously for 2 hours. The resulting solution was centrifuged and filtered through glass wool. Hyaluronic acid was precipitated by pouring the solution into 12 liters of cold acetone. The stringy material was washed three times with cold acetone, filtered, washed twice with alcohol and anhydrous ether. It was finally desiccated over P₂O₅ for 24 hours. This procedure gave a yield of 6 per cent of a product which gave a relative viscosity of 3.0 at a concentration of 10 mg. per ml. For the turbidity assay it was made up to 3.0 mg. per ml. in a 0.3 M phosphate buffer at pH 5.5.

The solution so prepared is slightly opalescent, but can be obtained water-clear by filtration through a Seitz filter. It is diluted to give a standard turbidity. This usually requires about a 2:3 dilution.

**Acidified Horse Serum Albumin**—Crystalline horse serum albumin was prepared by the method of Kekwick (13). 1 gm. of purified albumin was dissolved in 1000 ml. of 0.1 M acetate buffer of pH 4.1 and the pH subsequently adjusted to 3.75 with 4 N hydrochloric acid. This solution keeps indefinitely at 4°. Fraction V of bovine albumin, obtained from Armour and Company, was found to work equally well.

**Enzyme**—Testicular hyaluronidase was prepared from beef testes by the method of Hahn (8) and for the most part the material carried through the first ammonium sulfate precipitation was used. Enzyme so prepared was dialyzed free of sulfate at 4°, lyophilized, and stored in the dry state.
evidence of loss of activity has been observed in 6 months storage at -20°. For use it was dissolved in 0.2 M borate buffer of pH 7.5. The activity of such preparations was approximately 250 viscosity-reducing units per mg. of N.

Activity of enzyme was ascertained according to Haas (4) and expressed as the reciprocal of the half life $\times 10^3$. All determinations were carried out at 38°. Difficulty was encountered in attempting to standardize this unit, since it was found that the activity of the enzyme varied with different hyaluronic acid preparations. This was circumvented by correcting all activities to that obtained with a given lot of hyaluronic acid. In order to prevent undue introduction of new units in the literature, this same amount of enzyme was used as the arbitrary unit in the turbidity method to be described. The unit to be used in this discussion is then essentially based on a given amount of standard enzyme preparation.

Procedure

1 ml. of enzyme solution is mixed with 1 ml. of hyaluronic acid solution. (The enzyme has usually been made up in 0.5 ml. of 0.2 M borate buffer, pH 7.5, and mixed with 0.5 ml. of 0.9 per cent saline. The saline has been introduced into the procedure for convenience in the determination of hyaluronidase inhibitor.) This mixture is incubated for 45 minutes at 38° in Evelyn cuvettes. At the end of this time 10 ml. of acidified albumin reagent (at room temperature) are added and the mixture is shaken to insure complete mixing. Exactly 5 minutes later (by stop-watch) the tube is read in the Coleman junior spectrophotometer at a wave-length of 600 mp. Lower wave-lengths can be used, but 600 mp was selected to obviate interference of hemolysis in the determination of hyaluronidase inhibitor.

All determinations are done in duplicate. The tubes used are carefully selected so as to have a maximum variation of 0.5 of 1 scale division.

Experimental

Relationship of Optical Density to Hyaluronic Acid Concentration—Seastone (9) originally reported that the turbidity produced by a mixture of hyaluronic acid and acidified albumin is directly proportional to hyaluronic acid concentration. That this is so is confirmed by the results illustrated in Fig. 1. It will be seen that the relationship is linear up to about 2.0 mg. and beyond this the curve begins to decrease in slope. At higher concentrations a precipitate and finally a clot form.

Effect of pH on Turbidity Formation—The next group of experiments was performed to determine the optimum pH for the precipitation of the hyaluronic acid-albumin complex. A series of reagents was made up at different pH levels and the turbidity produced was determined. All other
conditions were kept constant in this experiment. The pH value given is that of the final mixture. It will be seen in Table I that turbidity development (expressed as optical density) is maximum at pH 3.82. This is achieved when the albumin reagent is adjusted to pH 3.75. If the pH is above or below this value, the turbidity is markedly decreased.

![Graph showing the relationship of hyaluronic acid concentration to optical density.](image)

**Fig. 1.** Relationship of hyaluronic acid concentration to optical density

<table>
<thead>
<tr>
<th>pH</th>
<th>Optical density</th>
</tr>
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<tbody>
<tr>
<td>2.15</td>
<td>0.153</td>
</tr>
<tr>
<td>3.10</td>
<td>0.274</td>
</tr>
<tr>
<td>3.42</td>
<td>0.293</td>
</tr>
<tr>
<td>3.82</td>
<td>0.337</td>
</tr>
<tr>
<td>4.20</td>
<td>0.305</td>
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</table>

**Table I**

*Effect of pH on Turbidity Development*

<table>
<thead>
<tr>
<th>Ionic strength</th>
<th>Optical density</th>
</tr>
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<tbody>
<tr>
<td>0.15</td>
<td>0.321</td>
</tr>
<tr>
<td>0.25</td>
<td>0.248</td>
</tr>
<tr>
<td>0.35</td>
<td>0.0303</td>
</tr>
<tr>
<td>0.45</td>
<td>0.0304</td>
</tr>
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</table>

**Table II**

*Effect of Variation of Ionic Strength on Turbidity Development*

Effect of Ionic Strength on Turbidity Development—It was found that turbidity development varied considerably with salt concentration. Table II shows the results of an experiment designed to assess the importance of this factor. It will be noted that the lowest ionic strength used was 0.15. It is inconvenient to go below this value and obtain proper conditions for the maintenance of pH both for enzymatic action and turbidity development. It is evident that the lowest salt concentration produced the maxi-
mum turbidity. As salt concentration increases, there is a marked drop in turbidity. The added salt was sodium chloride. No experiments were performed with other salts. As in previous experiments, all other conditions were kept constant.

Effect of Time of Reading on Turbidity—It was noted that turbidity decreases with time. Table III illustrates an experiment in which this factor was quantitatively evaluated. The first reading was taken at 5 minutes since this is the shortest interval that is convenient when a series of tubes is to be read. After longer intervals the rate of change decreases. Routine use of longer intervals was, however, found to be disadvantageous, since the differences in optical density between different concentrations of hyaluronic acid are decreased. It will be noted that the difference in optical density between 1.80 and 0.60 mg. of hyaluronic acid is 0.215 at 5 minutes, while at 30 minutes this had decreased to 0.172. This considerably decreases the sensitivity and range of the method.

Effect of Albumin Concentration on Turbidity Development—Since samples to be assayed may contain albumin, it was necessary to be sure that the albumin in the reagent is present in excess. This is particularly true when this method is applied to the assay of hyaluronidase inhibitor in blood serum. Fig. 2 shows the turbidity obtained with varying concentrations of albumin. It was found that the turbidity developed approaches a maximum at about 0.50 mg. per ml. It was decided to use 1.0 mg. per ml. routinely in order to assure an excess.

Relationship of Turbidity to Enzyme Concentration—In order to eliminate the necessity of multiple determinations, a method was devised, based on one turbidity determination. The experiment illustrated in Fig. 3 shows the relationship between turbidity (optical density) and enzyme concentration when enzyme has been incubated with hyaluronic acid for 45 minutes.

### Table III

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>1.80 mg.</th>
<th>1.40 mg.</th>
<th>1.00 mg.</th>
<th>0.60 mg.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.347</td>
<td>0.270</td>
<td>0.204</td>
<td>0.132</td>
</tr>
<tr>
<td>10</td>
<td>0.325</td>
<td>0.262</td>
<td>0.197</td>
<td>0.129</td>
</tr>
<tr>
<td>15</td>
<td>0.314</td>
<td>0.260</td>
<td>0.187</td>
<td>0.116</td>
</tr>
<tr>
<td>20</td>
<td>0.308</td>
<td>0.258</td>
<td>0.184</td>
<td>0.116</td>
</tr>
<tr>
<td>30</td>
<td>0.288</td>
<td>0.235</td>
<td>0.177</td>
<td>0.116</td>
</tr>
</tbody>
</table>
ASSAY OF HYALURONIDASE

It is seen that between 0 and 4 units this relationship is essentially linear. The region of linearity can be varied by changing the length of incubation.

![Graph showing the relationship of albumin concentration to optical density.]

**Fig. 2.** Relationship of albumin concentration to optical density

![Graph showing the relationship of hyaluronidase concentration to optical density.]

**Fig. 3.** Relationship of hyaluronidase concentration to optical density

With longer time intervals the method becomes more sensitive with less range, while with shorter time intervals the method becomes less sensitive but with greater range.
Careful attention must be paid to details of pH, salt concentration, and time. Different batches of hyaluronic acid vary in purity, but can be used to get comparable results simply by adjusting the concentration to a given optical density.

**Effect of pH on Activity of Hyaluronidase**—The next group of experiments was performed to determine the effect of variation of pH on the activity of hyaluronidase under these conditions. Several investigators have shown that the effect of salt concentration varies with pH and conditions of assay (3, 5). A series of phosphate buffers (0.3 M) was made at various pH levels and comparison of activity was made. All other conditions were kept constant. Table IV illustrates the results of these experiments. The $k_{v0}$ shown was calculated according to a method devised in this laboratory (12). It will be noted that the peak is obtained in the region of pH 5.5.

<table>
<thead>
<tr>
<th>Table IV Effect of pH on Activity of Hyaluronidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH ..........</td>
</tr>
<tr>
<td>$k_{v0}$ ..........</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table V Effect of Ionic Strength on Hyaluronidase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ionic strength ..........</td>
</tr>
<tr>
<td>Initial turbidity ..........</td>
</tr>
<tr>
<td>2 units (45 min.) ..........</td>
</tr>
<tr>
<td>4 &quot; (45 &quot; ) ..........</td>
</tr>
</tbody>
</table>

It is of interest that activity drops more rapidly on the alkaline side of the maximum than on the acid side. This pH (5.5) was then adopted as the standard in subsequent studies.

**Effect of Ionic Strength on Hyaluronidase Activity**—Several investigators have studied the effect of variation of salt concentration on hyaluronidase activity. This was found to vary with pH. It seemed desirable to redeetermine this by the method described in this paper. For this purpose a series of hyaluronic acid solutions in phosphate buffers of varying ionic strength at constant pH was used. All solutions were adjusted so that the optical density of the turbidity developed was constant. All other solutions were as described under "Procedure." Table V shows the results of such an experiment. In this only one reading was made; namely, after 45 minutes with 2 and 4 units. The results are expressed in terms of optical density and final ionic strength of the reaction mixture. Thus the borate
buffer, sodium chloride, and phosphate buffer are included in calculation of
the ionic strength. It will be noted that increasing ionic strength causes a
decrease in the activity of the enzyme in the range studied. It is imprac-
tical to use an ionic strength below 0.15 and have suitable conditions for the
maintenance of pH. When ionic strength was varied by using a constant
amount of phosphate and varying amounts of sodium chloride, similar
results were obtained.

It was decided to use a total ionic strength of 0.26 (hyaluronic acid is
made up in 0.3 M phosphate buffer). Although this does not represent the

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Enzyme units</th>
<th>Activity as optical density</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.284</td>
<td>0.164 0.000 0.034</td>
</tr>
<tr>
<td>2</td>
<td>0.280</td>
<td>0.174 0.080 0.031</td>
</tr>
<tr>
<td>3</td>
<td>0.284</td>
<td>0.182 0.093 0.039</td>
</tr>
<tr>
<td>4</td>
<td>0.296</td>
<td>0.181 0.103 0.040</td>
</tr>
<tr>
<td>5</td>
<td>0.288</td>
<td>0.185 0.101 0.046</td>
</tr>
<tr>
<td>Mean</td>
<td>0.287</td>
<td>0.177 0.0935 0.038</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.00625</td>
<td>0.00845 0.0058</td>
</tr>
<tr>
<td>S.D., enzyme units</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coefficient of variation, %</td>
<td>0.18 0.21 0.25</td>
<td>9.0 5.3 4.2</td>
</tr>
</tbody>
</table>

optimum ionic strength, it is the minimum concentration of buffer which
will maintain pH when various unknown samples are being analyzed.

Reproducibility—Table VI illustrates a series of experiments in which
repeated determinations of a standard enzyme preparation were made.
Each determination represents the average of two tubes. Two experi-
ments were run by one operator and three by another. It will be noted
that good agreement is obtained at all levels. The standard deviation in
terms of units of enzyme was obtained by constructing a curve of the means
and determining the variations of the individual experiments at each level.

The standard deviation in terms of optical density closely approximates
the accuracy of the Coleman junior spectrophotometer, indicating that
further changes in technique are unlikely to increase precision unless some
other type of instrument is used.
DISCUSSION

The estimation of hyaluronidase by the viscosity method represents a tedious and time-consuming process. Previous investigators have proposed the use of a turbidity method. These have not gained general favor because of instability of reagents and lack of standardization. Exact conditions for the use of this assay have now been determined. The reagents used are stable and are easily reproducible.

The literature on hyaluronidase is complicated by the various units introduced by different investigators. Since these vary widely with conditions of pH and salt concentration as well as with purity of substrate, it seems unwise at the present time to introduce a new unit. Since hyaluronidase is a relatively stable enzyme, we have used an arbitrary unit based on a given amount of partially purified enzyme. This can be compared with any unknown enzyme by any method of assay.

SUMMARY

1. Conditions for the use of the turbidity method for the determination of testicular hyaluronidase have been studied in detail.
2. A simple, rapid, and accurate procedure has been devised which utilizes stable reagents.
3. It is proposed that pending further knowledge the unit of hyaluronidase be based on a given amount of standard enzyme.

BIBLIOGRAPHY

A TURBIDIMETRIC METHOD FOR THE
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Albert Dorfman and Melvin L. Ott